## REMARKS

Reconsideration and withdrawal of the claim rejections are requested in view of the amendments and remarks herein.

## I. STATUS OF CLAIMS AND FORMAL MATTERS

Claims 12-15 and 18-22 are under consideration in this application. Claim 12 has been amended to overcome the Examiner's objection. Claims 19-22 have been added; claims 16-18 have been amended to modify dependency. No new matter is added.

It is submitted that the claims, herewith and as originally presented, are patentably distinct over the prior art cited by the Examiner, and that these claims were in full compliance with the requirements of 35 U.S.C. §112. The amendments of and additions to the claims, as presented herein, are not made for purposes of patentability within the meaning of 35 U.S.C. §§§§ 101, 102, 103 or 112. Rather, these amendments and additions are made simply for clarification and to round out the scope of protection to which Applicants are entitled. Furthermore, it is explicitly stated that the herewith amendments should not give rise to any estoppel, as the herewith amendments are not narrowing amendments.

## II. THE DOUBLE-PATENTING REJECTION IS ADDRESSED

Claims 12-15 and 18 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 84-91, 93-95, 141-143, 149 and 150 of U.S.S.N. 09/760,574. The rejection is traversed.

Claims 84-95 of U.S.S.N. 09/760,574 are directed to a DNA vaccine comprising a plasmid that expresses an immunogen of a bovine pathogen and a cationic lipid containing a quaternary ammonium salt. However, the claims of U.S.S.N. 09/760,574 do not render obvious the immunogenic composition of claims 12-15 or the kit of claim 18.

Further, the issue of whether there is indeed double patenting is contingent upon whether the claims of the pending applications are indeed allowed. For example, claims 141-143, 149 and 150 of U.S.S.N. 09/760,574 have been withdrawn from consideration, and may be cancelled and pursued in a separate application. If, upon agreement as to allowable subject matter in this application, it is believed that there is still a double patenting issue, the necessary Terminal Disclaimer(s) will be filed at that time.

Accordingly, reconsideration and withdrawal of the double patenting rejection, or at least holding it in abeyance until agreement is reached as to allowable subject matter, are requested.

## III. THE REJECTION UNDER 35 U.S.C. §103 IS OVERCOME

Claims 12-15 and 18 were rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Klippmark *et al.* in view of Suzu *et al.* and in further view of Felgner *et al.* and Crowe *et al.* These references were also considered in light of the teachings of Wathen *et al.* and Babiuk *et al.* The rejection is traversed.

The instant invention relates to an immunogenic composition or vaccine against BPIV-3 comprising a DNA plasmid that expresses BPIV-3 HN protein, F protein or both HN and F proteins. As is demonstrated by the data presented in the attached Declaration by Dr. Jean-Christophe Audonnet under 37 C.F.R. §1.132, the claimed compositions are efficacious as vaccines in bovine animals. As is stated in the paragraph beginning on page 1, line 11, of the specification, a significant cause of viral spreading is via excretion by infected animals. The experiments that are summarized in Dr. Audonnet's Declaration show that excretion of the virus is dramatically reduced in vaccinated animals after challenge with BPIV-3. The reduction of viral excretion after vaccination reduces the risk of contamination between animals, and consequently, limits the spread of disease among livestock, solving a significant problem in the art. The fact that the plasmid DNA compositions of the invention can act as vaccines and confer protection is surprising and unexpected.

As pointed out by the Examiner, Klippmark does not teach a plasmid vaccine against BPIV-3. Klippmark does not even teach "that the BPIV-3 HN and F proteins are immunogenic against infection by the virus, and that vaccines comprising these subunits are known to confer protection against the virus in animals", as alleged on page 6 of the Office Action. What Klippmark does teach is the characterization of human and bovine PIV strains using monoclonal antibodies. Klippmark mentions vaccines against PIV-3 in the first paragraph; however, it does not provide any data, it simply cites other studies:

> Several studies have shown that animals may be protected with whole virus and subunit vaccines containing both human and bovine PIV3 components (Morein *et al.*, 1983; Ray *et al.*, 1985). The two surface glycoproteins HN and F are important for the establishment of protection against disease (Morein *et al.*, 1983; Spriggs *et al.*, 1987). A mixture of both surface glycoproteins, HN and F (Ray *et al.*, 1988*a*), can induce protection against PIV3, as do HN and F proteins expressed by vaccinia virus and baculovirus recombinants (Spriggs *et al.*, 1987; van Wyke Coelingh *et al.*, 1987 [sic]).

The Morein reference (copy enclosed) shows that HN and F proteins, administered as multimeric micelles, elicited an immune response in mice, but not in lambs unless an adjuvant

was added to the preparation. On page 1567, the authors state that they do not know why the adjuvant was necessary in lambs, but hypothesize that "it may be due to species differences". Therefore, they were clearly unable to extrapolate their results from rodents to ruminants. They also state, on the same page, that "the problem of efficient presentation of the antigen remains". So, not only are recombinant DNA vaccines not taught or suggested in Morein, the authors were unable to demonstrate ideal, or even consistent results using subunit vaccines.

Ray (1985; abstract enclosed) showed an immune response in hamsters immunized with a combination of HN and F proteins, but not with inactivated whole virus that also contains HN and F proteins. As was true with Morein, predictable results were not obtained in hamsters by Ray. Given the potential interspecies variability demonstrated by Morein, there is no reason to think that either the teachings of Morein or Ray could be extended to bovine animals.

Spriggs *et al.* (copy enclosed) deals with the inoculation of cotton rats with recombinant HN and/or F proteins, prepared from cells infected with vaccinia virus. Vaccinia virus was used in this study simply as a mechanism to produce recombinant proteins. In the last sentence of Spriggs, the authors state that their study could be a useful foundation for further investigations, such as "for testing the general usefulness of live recombinant viruses as vaccines". So not only does Spriggs not teach how to use a recombinant virus as a vaccine, it states that the feasibility of doing so was in doubt. Furthermore, there is no teaching or even suggestion in Spriggs that a DNA plasmid vaccine would be effective in an immunogenic composition or vaccine against BIV-3. Similarly, van Wyke Coelingh *et al.* (abstract enclosed) showed an immune response in cotton rats inoculated with recombinant HN protein produced in baculoviral vectors. In addition to not teaching or suggesting the instant invention, as discussed above, the efficacy of the same composition may vary between rodents and bovines.

As discussed above, the attached Declaration by Dr. Jean-Christophe Audonnet provides data demonstrating that similar immune responses are elicited in cattle vaccinated with the currently claimed immunogenic composition or vaccine, when compared with a commercially available live attenuated virus vaccine. This evidence clearly shows that the claimed compositions are efficacious in the relevant animals. Given the failure of the art to teach a plasmid DNA vaccine against BPIV-3 that is effective in bovines, these results are surprising and unexpected.

In view of the above, it is submitted that Klippmark does not teach "that the BPIV-3 HN and F proteins are immunogenic against infection by the virus, and that vaccines comprising these subunits are known to confer protection against the virus in animals", as is stated on page 6 of the Office Action. At best, Klippmark only refers to studies that relate to the use of PIV-3 HN and F proteins in vaccines that were tested on rodents. The references cited by Klippmark, particularly Morein *et al.*, provide no expectation of success in larger animals. Further, these references cannot be extrapolated to the instant invention because there is no indication that the results achieved by administration of a protein-based vaccine correlate with those of administration of a DNA plasmid-based vaccine.

While Suzu *et al.* supplies the RNA and deduced amino acid sequences of BPIV-3 HN and F proteins, it does not remedy any of the deficiencies of Klippmark.

Further, Felgner provides broad suggestions regarding the potential use of DNA plasmids as vaccines; however, Felgner does not teach plasmids encoding BPIV-3 proteins. In addition, the only *in vivo* data relating to viral antigens provided by Felgner deals with HIV proteins expressed in mice. As discussed above, results obtained in mice cannot be extrapolated to larger animals, particularly in the complicated area of immune response. Also, Felgner does not teach or suggest the use of plasmids in eliciting an immune response against BPIV-3, which is of a completely different genera, family and order than HIV. (BPIV-3 is a paramyxovirus, whereas HIV is a lentivirus.) There is no indication, teaching or suggestion that any of the experiments in Felgner can be applied to the instant invention.

Crowe provides a non-enabling report of studies conducted by other parties. The section dealing with DNA immunization teaches away for the instant invention by suggesting that vaccination with plasmids expressing influenza A virus proteins "might prove useful in immunization strategies for RSV and PIV3" (p. 419). No plasmid construction or result of vaccination of bovines against BPIV-3 is described in this document.

Babiuk *et al.* relates to herpesvirus-1, and is not relevant. Wathen also teaches away from the instant invention, as it advocates the use of a glycoprotein that is a chimera between HN and F to improve a vaccine.

The Examiner is respectfully reminded that "obvious to try" is not the standard for formulating a *prima facie* case of obviousness under 35 U.S.C. §103. *In re Fine,* 5 U.S.P.Q. 2d 1596, 1599 (Fed. Cir. 1988). Also, the Examiner is additionally respectfully reminded that for

the Section 103 rejection to be proper, both the suggestion of the claimed invention and the expectation of success must be founded in the prior art, and not Applicants' disclosure. *In re Dow*, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988).

In this case, the Office Action relies on the combination of six different references, some of which do not teach anything at all, but rather, report results obtained by other researchers. Although it may have been obvious to try a DNA plasmid vaccine against BPIV-3, given the variability between viruses and the incongruous results obtained from one animal species to the next, there has been no demonstration by the Examiner that the skilled artisan could reasonably have expected success in bovines using an immunogenic composition or vaccine containing a plasmid with DNA encoding BPIV-3 HN and/or F proteins. The fact that the inventors of the instant application have been able to demonstrate efficacy is surprising and unexpected.

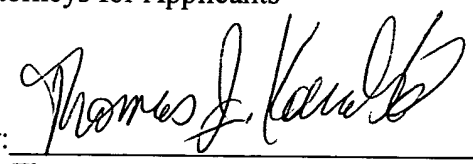
Although the cited references have been dealt with somewhat individually above, the failings of the cited references to predict, teach or suggest the success of the claimed invention are not remedied by combination with one another. It is therefore submitted that the claimed invention is not obvious over the cited references. Therefore, reconsideration and withdrawal of the 35 U.S.C. §103 rejection are respectfully requested.

## CONCLUSION

In view of these amendments, remarks, and the Declaration under 37 C.F.R. §1.132, the application is believed to be in condition for allowance. Early and favorable reconsideration of the application, reconsideration and withdrawal of the rejections, and prompt issuance of a Notice of Allowance are earnestly solicited.

Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP
Attorneys for Applicants

By: 

Thomas J. Kowalski
Reg. No. 32,147
Tel (212) 588-0800

*J. gen. Virol.* (1983), 64, 1557–1569. *Printed in Great Britain*



Key words: *protein micelles/immunogenicity/antigens/enveloped viruses*

# Protein Subunit Vaccines of Parainfluenza Type 3 Virus: Immunogenic Effect in Lambs and Mice

By BROR MOREIN,[1] MIKE SHARP,[2] BO SUNDQUIST[1] AND KAI SIMONS[3]*

[1] *Department of Virology, Faculty of Veterinary Medicine, National Veterinary Institute, Biomedical Center, Uppsala, Sweden,* [2] *Moredun Institute, Edinburgh, U.K. and* [3] *European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany*

(*Accepted 17 February 1983*)

## SUMMARY

Protein subunit vaccines were prepared from a mixture of the haemagglutinin (HN) and fusion (F) glycoproteins of parainfluenza type 3 virus (PI-3). The glycoproteins were isolated in three different forms and characterized by their sedimentation coefficients: 30S protein micelles (a complex of several HN and F glycoproteins devoid of detergent and lipid), 18S protein–TX complexes (a complex of several glycoproteins containing the detergent Triton X-100), and 4S protein–TX complexes (probably monomers of the glycoproteins complexed to Triton X-100). These preparations were tested as vaccines in mice and lambs. The immune response in the mice was assayed both in the serum and in extracts from the lungs using an ELISA technique. Both of the multimeric complexes were highly immunogenic. The 30S protein micelles induced a high antibody response after two injections with either 10 or 1 μg protein. The serum IgG titres reached levels of about 90 μg/ml and 40 μg/ml respectively. Similar titres were reached with the 18S protein–TX complexes. After two injections of either the 30S or the 18S complexes IgA antibody responses were detected in the lung extracts. The 4S protein–TX complexes were poor immunogens and induced low antibody responses in mice. The lambs were vaccinated with the 30S protein micelles, and the immune response was evaluated serologically and in challenge experiments. The 30S protein micelles in an oil adjuvant induced detectable serum antibody titres as well as protective immunity against the pneumonia caused by the PI-3 virus.

## INTRODUCTION

The effects of vaccines against virus diseases may be hampered by adverse reactions caused by components in the vaccines not necessary for induction of immunity. The safety of virus vaccines can be considerably improved by purification of the essential viral antigens. For enveloped viruses the surface glycoproteins seem to be the main antigens required to induce a protective immune response (Bachmayer *et al.*, 1976; Cox *et al.* 1977; Hilleman, 1976; Hunsmann *et al.*, 1981; Merz *et al.*, 1980), but so far a generally applicable method for preparing effective vaccines from isolated surface proteins is lacking. Previous methods using a combination of organic solvents and detergents (e.g. Tween–ether) have given variable results (Ginsberg, 1975; Gross *et al.*, 1977). Methods based on detergent extraction alone have proved more efficient (Bachmayer *et al.*, 1976; Brady & Furminger, 1976). Our own studies have shown that the physical form of a membrane antigen has a considerable influence on its immunogenicity. We isolated the surface glycoproteins of Semliki Forest virus in three different forms using mild non-denaturing conditions (Helenius & Simons, 1975): (i) as protein monomers (complexed to detergent), (ii) as protein micelles (soluble spike protein octamers virtually devoid of lipid and detergent), and (iii) as virosomes, in which the glycoproteins were reconstituted into liposomes of egg lecithin. When these preparations were tested as vaccines against the lethal encephalitis caused by the virus in mice, it was found that the multimeric forms of the protein,

0022-1317/83/0000-5546 $02.00 

the protein micelles and the virosomes gave exceptionally efficient protection whereas the protein monomers were ineffective (Morein *et al.*, 1978).

We are now extending our studies on the correlation of immunogenicity with antigen presentation to parainfluenza type 3 (PI-3) virus, a paramyxovirus. This virus causes local infections in the respiratory tract of man, lambs and in other animals. We have prepared different forms of the surface glycoproteins, the haemagglutinin/neuraminidase (HN) and the fusion (F) proteins and tested their immunogenic properties in mice and lambs. Our results show that protein micelles (the multimeric forms) are efficient immunogens in mice. In lambs an adjuvant had to be added to induce a protective immune response. Some of the results have been reported previously in a preliminary form (Simons *et al.*, 1980; Morein *et al.*, 1981).

## METHODS

*Virus growth and purification.* Two strains of PI-3 virus were used. The Umea 23 strain was propagated in secondary calf kidney cells using Hanks' balanced salt solution with 0·5% lactalbumin hydrolysate, 2% horse serum and antibiotics, and this strain was used for the experiments with mice. The G2 strain was propagated in secondary foetal lamb kidney (Flk) cells using Eagle's minimal essential medium with 10% lactalbumin hydrolysate, 0·5% foetal bovine serum and antibiotics, and this virus was used for the experiments with lambs. The virus was concentrated by hollow-fibre filtration (Amicon DC-2; exclusion limit 100000 daltons), and purified by centrifugation through 30% (w/w) sucrose in 0·05 M-Tris–HCl pH 7·4, containing 0·1 M-NaCl (TN) onto a cushion of 60% (w/w in TN) sucrose for 40 min at 4 °C and 25000 rev/min in a SW27 Beckman rotor. The sucrose was removed by suspending the virus-containing fractions in TN followed by pelleting at 25000 rev/min for 40 min at 4 °C in a SW27 Beckman rotor. The pellet was resuspended in TN to a concentration of about 10 mg/ml. The amount of virus protein was determined by the Lowry method (Lowry *et al.*, 1951) with 3·5 mM-SDS in the reaction mixture. The surface glycoproteins of the virus were labelled with the galactose oxidase–[$^3$H]borohydride procedure (Luukkonen *et al.*, 1977). The labelled PI-3 virus was separated from non-bound [$^3$H]borohydride by centrifugation through 30% (w/w) sucrose onto a cushion of 60% (w/w) sucrose.

*Isolation of PI3 virus surface glycoprotein preparations*

*Protein micelles.* The PI-3 protein micelles containing the virus surface glycoproteins, HN and F proteins were prepared essentially as described by Helenius & von Bonsdorff (1976) and Simons *et al.* (1978). About 1 mg of PI-3 virus in TN was solubilized, with 2% Triton X-100 together with $^3$H-labelled virus. A sample volume of about 200 µl was layered onto 300 µl 15% sucrose containing 1% Triton X-100 and TN over a 12 ml sucrose gradient in TN ranging from 20 to 50% (w/w). The centrifugation was performed at 40000 rev/min for 22 h at 20 °C. Fractions of 500 µl were collected from the bottom and aliquots were measured for radioactivity. The pooled fractions were dialysed against 0·005 M-Tris–HCl, 0·01 M-NaCl, pH 7·4, for 20 h in the cold, and concentrated by lyophilization. This preparation had a sedimentation coefficient of about 30S (see later) and is referred to as the 30S protein micelles. In some of the vaccination experiments 30S protein micelles were mixed with an equal volume of mineral oil adjuvant (Bayol-Falba).

*PI-3 virus surface glycoproteins complexed to Triton X-100.* PI-3 virus was solubilized with Triton X-100 as described above, and centrifuged for 20 h at 39000 rev/min at 4 °C through a sucrose gradient composed of a layer of 200 µl 8% sucrose containing 1% Triton X-100 in TN on top of a 12 ml 10 to 30% sucrose gradient containing 0·05% Triton X-100. About 50% of the HN and F glycoproteins sedimented in a peak with a sedimentation coefficient of about 18S and the other 50% sedimented much slower, at about 4S. The 18S complex is referred to as the 18S protein–TX complex (PI-3 virus glycoprotein complexed to Triton X-100) and the 4S complex is designated the 4S protein–TX complex.

*Other methods.* Polyacrylamide gel electrophoresis was done in slab gels of 10% acrylamide, and 0·27% *N,N'*-methylenebisacrylamide in the presence of SDS in a discontinuous buffer system (Laemmli, 1970). The samples were prepared with 1% SDS and 1% 2-mercaptoethanol and heated to 100 °C for 1 min.

The PI-3 surface glycoproteins were isolated by lentil lectin chromatography. The HN and F polypeptides were solubilized by suspending the PI-3 virus (1 to 2 mg/ml) in 2% Triton X-100 in TN at room temperature for 60 min and then applied to a column of Lentil–Sepharose 4B (Pharmacia) equilibrated with TN buffer containing 0·5% Triton X-100. After the column had been washed with 5 bed vol. of equilibration buffer, the immobilized glycoproteins were eluted with 2·5% methyl-α-D-mannoside dissolved in TN buffer.

The surface glycoprotein preparations isolated by sucrose density gradient centrifugation were characterized by sedimentation velocity analysis as described by Martin & Ames (1961) using 29S Semliki Forest virus protein micelles, 19S thyroglobulin, and 7S IgG as standards. Electron microscopy of negatively stained preparations was done as described by Simons *et al.* (1978). The amount of actin was measured by the DNase inhibition assay (Blikstad *et al.*, 1978).

Table 1. *Vaccination of mice with parainfluenza 3 virus glycoproteins*

| | Group | No. of mice | Week 0 1st vaccination | Week 3 2nd vaccination | |
|---|---|---|---|---|---|
| (*a*) | A | 10 | 10 μg 30S protein micelles | 10 μg 30S protein micelles | Serum samples were collected regularly (Fig. 5) until the end of the experimental period, 6 weeks after the 2nd vaccination* |
| | B | 10 | 1 μg 30S protein micelles | 1 μg 30S protein micelles | |
| | C | 10 | 0·1 μg 30S protein micelles | 0·1 μg 30S protein micelles | |
| | D | 10 | 10 μg 18S protein–TX complexes | 10 μg 18S protein–TX complexes | |
| | E | 10 | – | – | |
| (*b*) | F | 10 | 5 μg 4S protein–TX complex | 5 μg 4S protein–TX complex | Serum samples were collected regularly (Fig. 6) until the end of the experimental period, 6 weeks after the 2nd vaccination |
| | G | 10 | 5 μg 18S protein–TX complex | 5 μg 18S protein–TX complex | |
| | H | 10 | Mixture of 4S and 18S protein–TX complexes: 2·5 μg of each | Mixture of 4S and 18S protein–TX complexes: 2·5 μg of each | |

* Extracts were collected from lung and trachea following necropsy of the mice, 6 weeks after the 2nd vaccination.

Table 2. *Vaccination of lambs with parainfluenza 3 virus*

| Group | No. in group | Treatment at weeks after vaccination: 0 | 3 | 6 | 7 |
|---|---|---|---|---|---|
| A | 4 | Unvaccinated | Unvaccinated | PI-3 virus: $10^{9.3}$ $TCID_{50}$, intranasal + intratracheal | Kill and necropsy |
| B | 7 | 15 μg micelles TN buffer, intramuscular | 15 μg micelles TN buffer, intramuscular | | |
| C | 6 | 15 μg micelles Bayol-Falba, intramuscular | 15 μg micelles TN buffer, intramuscular | | |

*Experimental animals*

*Lambs.* Hysterectomy-derived, colostrum-deprived lambs were reared under specific pathogen-free (SPF) conditions and allocated to groups as required.

*Mice.* BALB/c male mice were used. They were 6 weeks old at the start of the experiment and were obtained from Bomholtgard Ltd, Ry, Denmark.

*Experimental designs*

*Vaccination experiments in mice.* The mice were kept in cages of five, allocated to groups of ten and treated according to the experimental protocol (Table 1 *a, b*). The mice were injected twice with an interval of 3 weeks subcutaneously with different doses of the PI-3 glycoprotein preparations. Blood samples were collected at weekly intervals by bleeding the mice from the retrobulbar plexus. Serum was prepared and stored at −20 °C until used. The mice were exsanguinated and killed 4 or 6 weeks after the second vaccination. The lungs were removed and the immunoglobulins were extracted in TN at the proportion of 1 ml buffer to 1 g trachea or lung tissue (Waller *et al.*, 1980).

*Vaccination experiments in lambs.* Seventeen SPF lambs were used in the first experiment (Table 2) and 25 in the second. The lambs were injected intramuscularly with 1 ml of the respective vaccines. Blood samples were taken before vaccination and at weekly intervals thereafter until the end of the experiment. Sera were stored at −20 °C until examined. Three weeks after the second vaccination, each lamb was injected intratracheally with 8 ml and intranasally with 2 ml of the G2 strain of PI-3 virus ($10^{8.3}$ $TCID_{50}$/ml, experiment 1; $10^{7.7}$ $TCID_{50}$/ml, experiment 2). Nasopharyngeal swabs for virus isolation were obtained daily from each lamb, held in transport medium on ice and inoculated onto Flk cells within 2 h. Swabs were then stored at −70 °C and positive samples

were titrated retrospectively, as described previously (Wells *et al.*, 1978). The lambs were killed 7 days after challenge by intravenous pentobarbitone and exsanguinated by severing the axillary vessels. Lung lesions, as viewed from the dorsal aspect, were recorded on lung diagrams (Wells *et al.*, 1978).

*Immunological tests.* The haemagglutination inhibition (HI) test was performed as described previously (Wells *et al.*, 1976).

The enzyme-linked immunosorbent assays (ELISA) to detect antibodies in mouse and sheep sera differed. The ELISA for mouse antibodies employed Dynatech M129B plates coated for 1 h at 37 °C with 100 µl/well of the Umea 23 strain of PI-3 virus (0·2 µg/ml). After three washings with phosphate-buffered saline (PBS) containing 0·05% Tween 20, 100 µl of the test sera diluted 1/1000 or 1/5000 or the extracts from lungs or tracheae diluted 1/500 were added to each well and the plates were incubated for 1 h at 37 °C. After three washings the plates were incubated for 1 h at 37 °C with 100 µl/well of a rabbit anti-mouse IgG (2 µg/ml) (Litton Bionetics Laboratory Products, Kensington, Md., U.S.A.), or with rabbit anti-mouse IgM or IgA, both 2 µg/ml (Miles Laboratories). After three washings, 100 µl of swine anti-rabbit immunoglobulin (8 µg/ml) conjugated with horseradish peroxidase (National Veterinary Institute, Stockholm, Sweden) was added and the plates were incubated for 1 h at 37 °C. After three washings, 100 µl of the substrate 5-aminosalicylic acid was added to the wells and the colour reaction was read at 490 nm after incubation for 60 min at room temperature. The absorbance values shown were corrected by deducting the mean values of samples from non-vaccinated control mice.

The amount of antibody was quantified according to the procedure of McLean *et al.* (1980).

The ELISA for detecting antibodies from sheep was performed in Dynatech M129A plates coated overnight at 4 °C with 100 µl/well of the G2 strain of PI-3 virus (8 µg/ml). A single buffer was used as diluent and washing fluid throughout the remainder of the test (Hommers *et al.*, 1982). After three washings, 100 µl of the 1/150 diluted test sera were added to the plates and incubated for 2 h at 37 °C. After a further three washings, 100 µl of a rabbit anti-sheep immunoglobulin (0·3 µg/ml) conjugated with alkaline phosphatase was added to each well and incubated at room temperature for 3 h. The plates were washed three times, 100 µl of substrate was added (*p*-nitrophenyl phosphate) and the colour reaction determined at 405 nm after incubation for 1 h at room temperature.

## RESULTS

### *Preparation and characterization of the PI-3 virus surface glycoprotein complexes*

The polypeptide pattern of the purified PI-3 virus preparation is shown in Fig. 1(*a*). The pattern is similar to that described for other parainfluenza viruses and to that previously described for PI-3 virus by Shibuta *et al.* (1979). The protein band with an apparent mol. wt. of 44000 is actin. The DNase inhibition assay indicated that 1 to 3% of the protein content was actin. The 73000 and the 51000 mol. wt. bands we assign (by analogy with other paramyxoviruses) as the HN and the F glycoproteins respectively (see Choppin & Compans, 1975). These could be isolated by lentil lectin chromatography (Fig. 1*b*) and were the only polypeptides labelled with the galactose oxidase–[$^3$H]borohydride procedure (not shown).

Galactose oxidase–borohydride $^3$H-labelled PI-3 virus was solubilized with Triton X-100 and applied to a sucrose gradient designed for isolation of protein micelles. After centrifugation, the radioactivity was distributed in two peaks, one in the lower, detergent-free portion of the gradient (Fig. 2*a*). SDS–polyacrylamide gel electrophoresis showed that the HN and the F polypeptides were present in the lower peak (Fig. 2*b*, lane 2). The radioactivity on the top of the gradient probably represents $^3$H-labelled glycolipids since little protein was present. The non-glycosylated polypeptides were found in the pellet (Fig. 2*b*, lane 3). When only trace amounts of virus were applied to the gradient, no clear-cut peaks were seen and the radioactivity was smeared throughout the gradient.

The peak fractions 6 to 10 in Fig. 2(*a*) were pooled and concentrated by lyophilization. The sedimentation coefficient of the protein was determined by velocity sucrose gradient centrifugation. The average value was about 30S.

We tested whether the PI-3 glycoprotein complexes were influenced by the lyophilization procedure used to concentrate the protein. A preparation of glycoprotein complexes obtained from 1 mg of PI-3 virus was divided into two halves. One portion was concentrated by vacuum dialysis in a collodion bag (SM 13200 Sartorius membrane filter) and the other was dialysed against 5 mM-Tris–HCl, 0·015 M-NaCl, pH 7·4 to remove the sucrose. The dialysed material was lyophilized and redissolved in water. The lyophilized protein complexes had the same

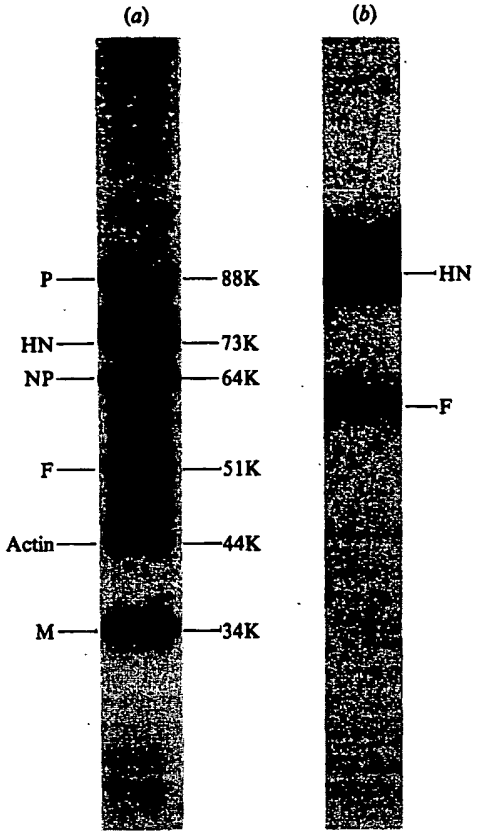



Fig. 1. Polyacrylamide gel electrophoresis of PI-3 virus (Umea 23 strain) proteins in the presence of SDS. (*a*) Purified whole virus. Their apparent molecular weights are indicated (K = mol. wt. × $10^3$). (*b*) HN and F glycoproteins isolated from the virus by lentil lectin chromatography. Protein stain was Coomassie Brilliant Blue.

distribution when analysed in a sedimentation velocity gradient as the material subjected to ultrafiltration (not shown). Furthermore, the lyophilization procedure gave a much higher recovery of the protein than did ultrafiltration, during which variable amounts of the protein were adsorbed to the walls of the collodion bag. From 1 mg of total PI-3 virus protein 0·13 mg of pure HN and F polypeptides could be recovered after lyophilization as protein micelles. After ultrafiltration the yields were about 50% lower.

Electron micrographs of negatively stained preparations of the PI-3-virus protein complexes showed a radial arrangement of spikes protruding from a central core (Fig. 3), probably formed by the association of hydrophobic protein domains (see Simons *et al.*, 1978). The overall diameter was 32 ± 3 nm. These complexes are similar in size and appearance to the glycoprotein micelles isolated from Sendai virus (Scheid *et al.*, 1972; Simons *et al.*, 1978).

When $^3$H-labelled PI-3 virus solubilized into Triton X-100 was centrifuged in a sucrose gradient containing Triton X-100 (the sucrose gradient in Fig. 2 did not contain detergent), two peaks containing the PI-3 virus surface glycoproteins were observed (Fig. 4*a*). The more rapidly sedimenting peak had a sedimentation coefficient of 18S, and was enriched in the HN glycoproteins (Fig. 4*b*, lane 3). The more slowly sedimenting peak had a sedimentation coefficient of about 4S, and was enriched in the F glycoprotein (Fig. 4*b*, lane 2). Recentrifugation of the 18S and 4S protein–TX complexes in sucrose gradients containing 0·05% Triton X-100 showed that they were stable and did not convert into each other (not shown).

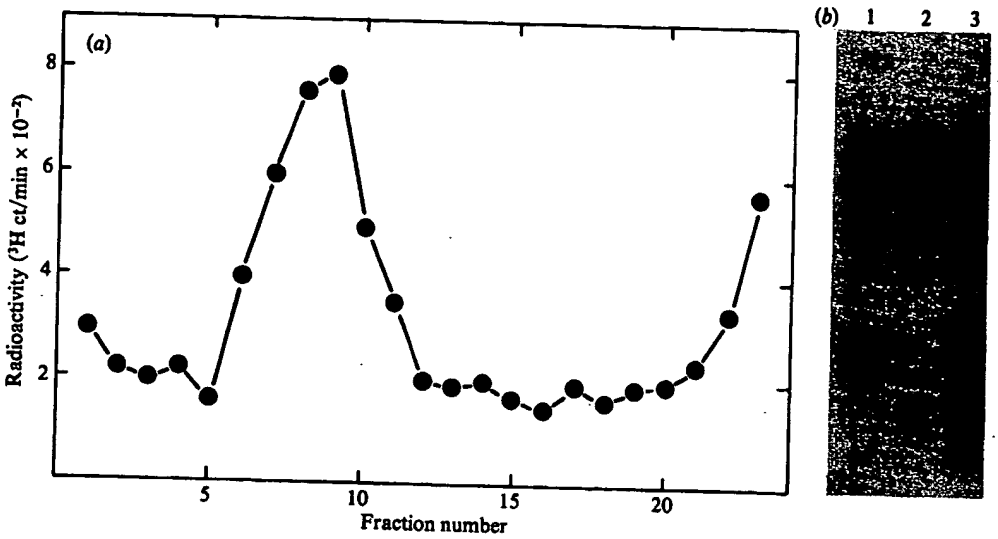



Fig. 2. (*a*) Preparation of the 30S protein micelles from PI-3 virus labelled with [$^3$H]borohydride. Sedimentation was towards the left. For details see Methods. (*b*) Polyacrylamide gel electrophoresis in the presence of SDS. Lane 1, PI-3 virus; lane 2, sample from the pooled fractions 6 to 10; lane 3, sample from the pellet. Stain was Coomassie Brilliant Blue.

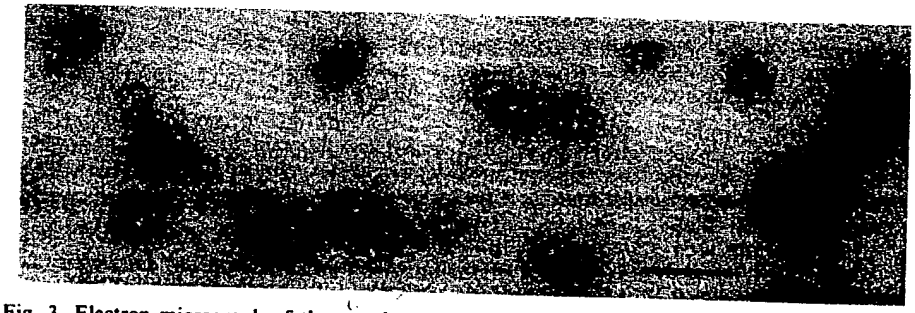


Fig. 3. Electron micrograph of the membrane glycoproteins of PI-3 virus isolated as 30S protein micelles. Bar marker represents 100 nm.

*Response of mice to vaccination*

We tested the immune response to the different preparations of the PI-3 virus glycoproteins in mice as experimental animals. The experimental protocol is given in Table 1. The antibody responses were measured with an ELISA technique weekly during the experimental period, or by haemagglutination inhibition at the end of the experimental period.

Both of the multimeric forms, the 30S protein micelles and the 18S protein–TX complexes induced a high immune response in the mice. One μg of the 30S protein micelles gave a detectable IgG response amounting to about 40 μg of antibodies per ml of serum after the second vaccination with the same dose (Fig. 5). Lower doses (0·1 μg) gave a much lower response. When the effect of a dose of 10 μg 30S protein micelles was compared to that induced by the same dose of 18S protein–TX micelles, the response to each preparation was found to be similar both in the IgM and IgG class of antibodies. The secondary response reached levels of about 100 μg IgG antibodies per ml of serum. The immune response as measured by HI assay 6 weeks after the second immunization correlated with that measured by the ELISA test, the maximum mean titres being 128. The immune response was also measured in extracts of lungs and trachea at the

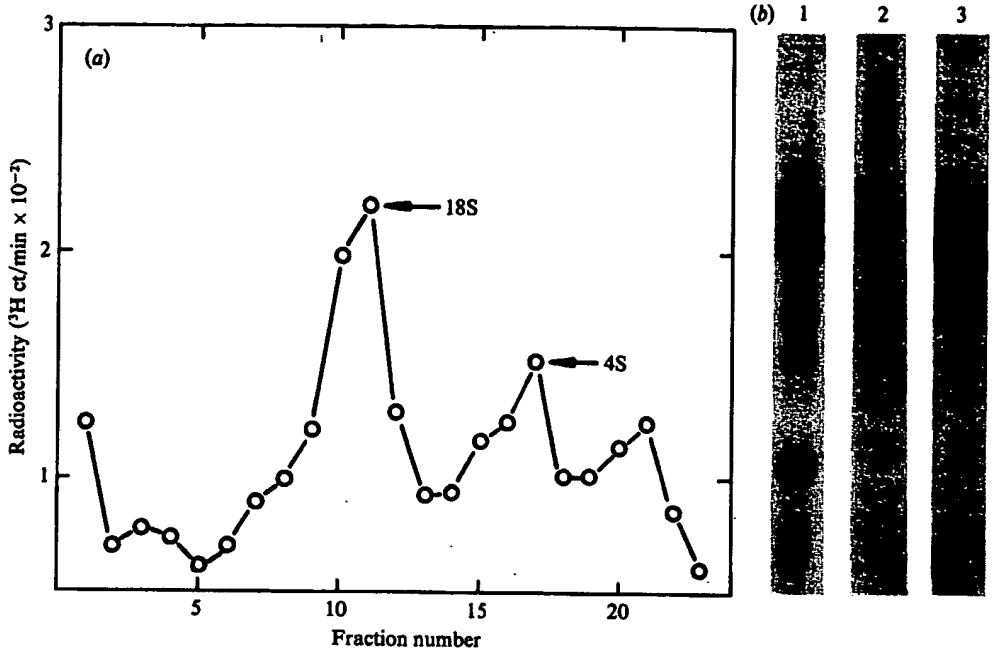



Fig. 4. (*a*) Preparation of the 18S and 4S protein–TX complexes from PI-3 virus labelled with [$^3$H]borohydride. Sedimentation is towards the left. For details see Methods. (*b*) Polyacrylamide gel electrophoresis in the presence of SDS. Lane 1, PI-3 virus; lane 2, sample from the 4S peak; lane 3, sample from the 18S peak. Stain was Coomassie Brilliant Blue.

end of the experimental period. An IgA response was detected in lung extracts from mice vaccinated with 1 and 10 μg of the 30S protein micelles and with 10 μg of the 18S protein–TX complexes.

Mice vaccinated with 5 μg of the 4S protein–TX complex showed no antibody response after the first vaccination (Fig. 6). After the second vaccination they responded with low titres (mean 8 μg IgG per ml of serum). A mixture of 4S protein–TX complexes with 18S protein–TX complexes seemed to lower the response to the multimeric spike protein form as was shown by mixing 2·5 μg of each of the two preparations prior to injection (Fig. 6). No response was detected after the first immunization, and after the second the response was as low as with 4S protein–TX complexes alone and clearly lower than after vaccination with 1 μg 30S protein micelles (Fig. 5). This shows that not only is the slowly sedimenting form of the virus surface glycoproteins poorly immunogenic, it also might suppress the antibody response to the multimeric form. The detergent itself has no suppressive effect since the 18S protein–TX complexes alone are highly immunogenic.

The experiments thus showed that both the 30S protein micelles and the 18S protein–TX complex are highly immunogenic in mice whereas the 4S protein–TX complex is a poor immunogen.

*Response of lambs to vaccination*

To test the efficiency of the PI-3 spike proteins in producing protective immunity in lambs, we used the virulent G2 strain to prepare the vaccine. We chose to focus on the 30S protein micelles since our previous studies with Semliki Forest virus had shown that this form of the spike proteins was an excellent vaccine. The first series of experiments was performed in SPF lambs with 30S protein micelles either alone or mixed with an oil adjuvant (Table 2).

Following the first vaccination only those lambs receiving micelles in adjuvant developed

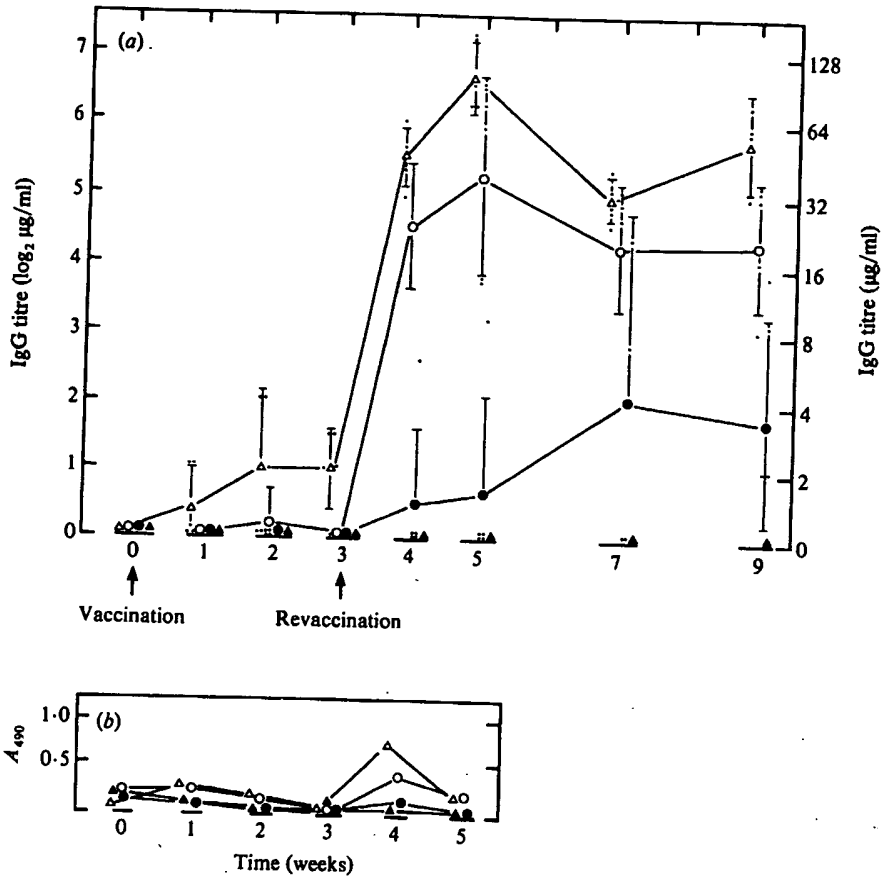



Fig. 5. Antibody response in serum from BALB/c mice, measured with an ELISA technique following immunization with 30S protein micelles as described in Table 1 (*a*). (*a*) Antibody response measured as the IgG class; (*b*) antibody response measured as the IgM class. △, 10 μg 30S micelles, mean values; ○, 1 μg 30S micelles, mean values; ●, 0·1 μg 30S micelles, mean values; ▲, non-vaccinated controls, mean values. Vertical lines show standard deviations and dots represent individual values.

antibodies detectable by ELISA or HI (Fig. 7 and 8). After the second vaccination, there was a further rise in antibody titres, and a slight increase in titre by ELISA in those lambs receiving micelles alone. Haemagglutination-inhibiting antibody was not stimulated in these lambs by vaccination. Challenge with live virus elicited a further rise in serum antibody levels in all lambs, this increase being particularly marked, by ELISA, in the animals vaccinated with micelles without adjuvant. In unvaccinated lambs low titres of antibody were detected after challenge.

*Response of lambs to challenge with PI-3 virus*

In this experiment (Table 2), the control lambs and lambs vaccinated with micelles without adjuvant developed a mild respiratory illness and pyrexia as described previously (Wells *et al.*, 1976). In contrast to this, the lambs vaccinated with micelles in adjuvant developed no signs of illness.

Virus was recovered from the unvaccinated control lambs for 6 days, with peak titres 3 to 5 days after inoculation (Fig. 9). Virus was excreted from most lambs that received micelles without adjuvant, for a similar period and achieved similar titres. However, of those lambs receiving micelles in adjuvant, only one lamb provided evidence of virus replication. This group

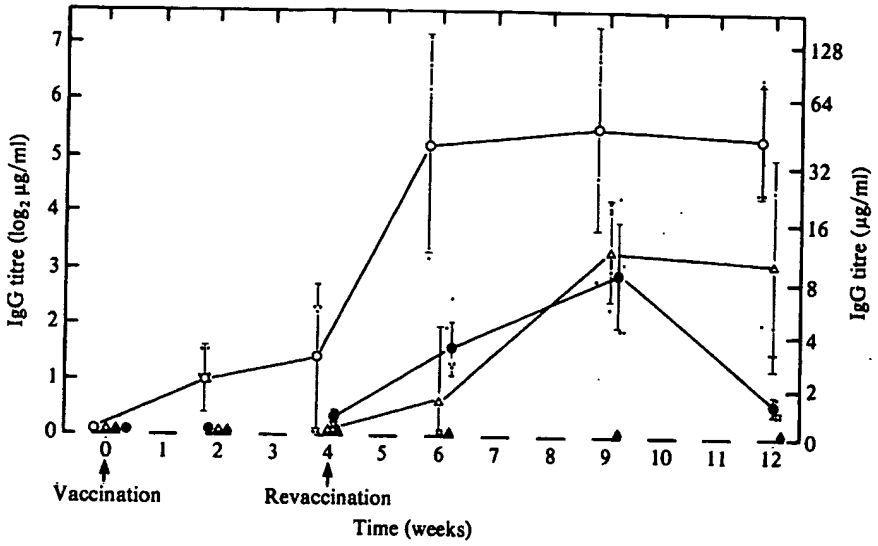



Fig. 6. Antibody response (mean values) in serum from BALB/c mice, measured with an ELISA technique following immunization with 5 µg 4S protein–TX complexes (●), 5 µg of the 18S protein–TX micelles (○) or 2·5 µg of the latter mixed with 2·5 µg of the 4S protein–TX complexes (△) as described in Table 1 (*b*); non-vaccinated controls are also shown (▲).

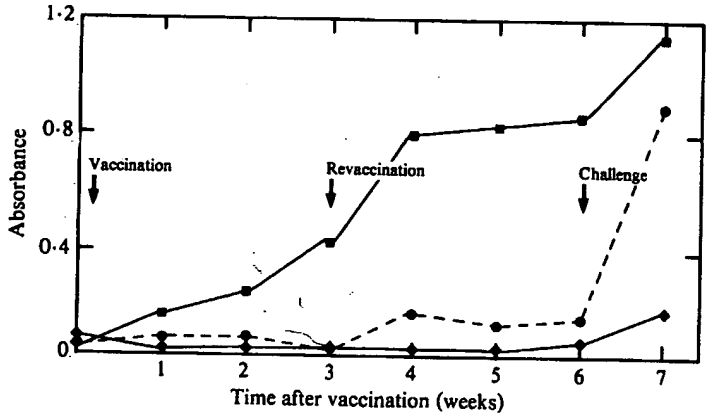



Fig. 7. Antibody response in the IgG class in serum from lambs, measured with an ELISA technique following immunization with the 30S protein micelles. The animals were immunized as described in Table 2. ◆, Mean value from four animals, non-vaccinated controls; ●, mean value from seven animals vaccinated twice with 30S protein micelles without adjuvant; ■, mean value from six animals vaccinated twice with 30S protein micelles. Adjuvant was included in the first immunization.

of lambs also showed reduced pneumonic lesions; two lambs had no lesions at all. The other vaccinated lambs had lesions as extensive as those in the control lambs.

One further experiment was done using 25 lambs to test whether higher doses of the 30S protein micelles alone induced a protective immune response. Two vaccinations of 25 and 50 µg protein respectively were given to two groups of lambs. The results were no better than with two doses of 15 µg 30S protein micelles alone. These experiments thus showed that a protective immune response could be induced in lambs by vaccination with the 30S protein micelles in oil adjuvant. Although lambs vaccinated with micelles without adjuvant developed only low titres of IgG antibodies and were not protected, the rapid rise in antibody titres following challenge with live virus indicated the priming effect of such vaccination.

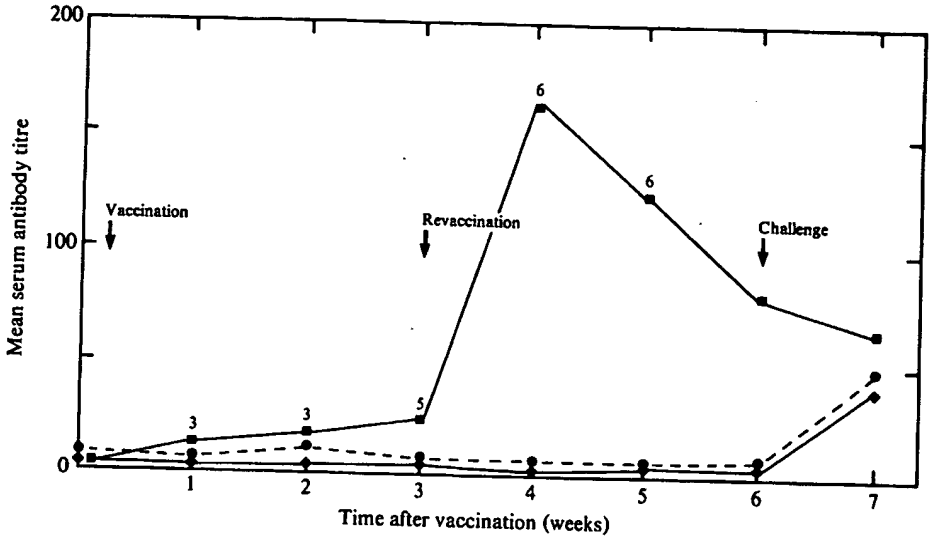



Fig. 8. Antibody response measured by haemagglutination inhibition in serum from lambs following immunization with the 30S micelles. The animals were immunized as described in Table 2. ◆, Mean value from four animals, non-vaccinated controls; ●, mean value from seven animals vaccinated twice with 30S protein micelles without adjuvant; ■, mean value from six animals vaccinated twice with 30S protein micelles. Adjuvant was included at the first immunization. The numbers in the figure indicate the number of lambs that became seropositive.

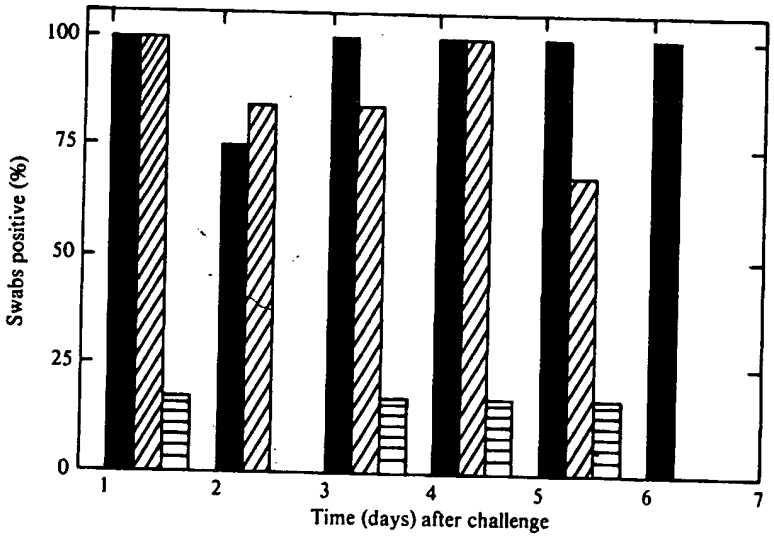



Fig. 9. Recovery of PI-3 virus from nasal swabs after challenge (see Table 2). Group A (■), non-vaccinated lambs; group B (▨), lambs vaccinated twice with 30S protein micelles without adjuvant; group C (▤), lambs vaccinated twice with 30S protein micelles. Adjuvant was included in the first immunization.

## DISCUSSION

With the possibility of producing the virus antigens responsible for induction of a protective immunity either by recombinant DNA technology or as synthetic antigens (Emtage & Carey, 1980; Kleid *et al.*, 1981; Lerner *et al.*, 1981; Muller *et al.*, 1982), the interest in protein subunit

vaccines has evidently increased. However, even if it does prove possible to produce the required antigens in large quantities, the problem of efficient presentation of the antigen remains. Not only is it uneconomical to use large doses for vaccination, but if the dose is too high the preparation might contain enough toxic components to cause adverse reactions, one of the problems one wants to avoid by the use of subunit vaccines. We have in a series of studies tried to analyse which form of virus surface glycoprotein is the most efficient as an immunogen. Our results with the PI-3 virus are essentially similar to those we earlier obtained with Semliki Forest virus (Morein *et al.*, 1978; Balcarova *et al.*, 1981). The monomer forms of the spike proteins are poor immunogens, whereas the multimer forms are highly immunogenic. In mice 0·1 μg of 30S protein micelles from the PI-3 virus gave almost as high an immune response as 5 μg of the 4S protein–TX complex. Moreover, it appeared that the 4S protein–TX complexes from PI-3 virus could suppress both the IgM and IgG responses against the virus surface glycoproteins when the mice were immunized at the same time with the highly immunogenic multimer form, the 18S protein–TX complex. The suppression could be due to the stimulation of T suppressor cells (see Gershon, 1974; Tada & Okumura, 1979) but more work is clearly needed to analyse this effect. This aspect is of importance since previous subunit vaccines against enveloped viruses have usually contained mixtures of protein micelles, protein monomers and lipids.

In lambs we did not obtain protection against pneumonia without including adjuvant in the first vaccination with the protein micelles prepared from the G2 strain of PI-3 virus. This was somewhat disappointing. In our previous experiments in mice with Semliki Forest virus, excellent protection was achieved with protein micelles alone. However, it should be emphasized that earlier studies with formalin-inactivated whole virus vaccines of PI-3 virus have shown that adjuvant also had to be included with those vaccines to achieve a protective response (Wells *et al.*, 1976). The reason for the need of adjuvant in lambs is not known, but it may be due to species differences or to different requirements for establishing mucosal immunity as compared to humoral immunity. We have not yet differentiated in detail the antibodies induced by the vaccinations to the two different virus glycoproteins. However, preliminary immunoblotting experiments show that sera from mice and lambs (with antibodies detectable by our ELISA assays) contained antibodies to both the HN and the F proteins (B. Morein & M. Sharp, unpublished results). Clearly, the qualitative nature of the immune response will influence the protection afforded by the vaccination (see Merz *et al.*, 1980).

The simple one-step procedure used to prepare micelles of membrane glycoproteins of PI-3 virus was originally devised for the Semliki Forest virus spike glycoproteins (Helenius & von Bonsdorff, 1976). In contrast to previous procedures to prepare micelles of virus spike proteins (Laver & Valentine, 1969; Scheid *et al.*, 1972), this method seems generally applicable to amphiphilic proteins, which have a fairly large hydrophilic domain outside the membrane and a small hydrophobic protein domain in the lipid bilayer. The procedure has been used by us to make micelles of the surface glycoproteins of Sendai virus, fowl plague virus and vesicular stomatitis virus (Simons *et al.*, 1978; Matlin *et al.*, 1981, 1982). The procedure has also been used by Heinz & Kunz (1980) to prepare protein micelles of the surface glycoproteins of tick-borne encephalitis virus, by Skelly *et al.* (1981) for the hepatitis B virus surface antigen, and, somewhat modified by Schneider *et al.* (1980) for the Friend leukaemia virus glycoproteins. One additional advantage of the procedure is that further purification of the relevant antigens is achieved during sucrose gradient centrifugation. Actin which was present in our PI-3 virus preparation was decreased to almost undetectable levels in the 30S protein micelles (Fig. 2). This might be important since host components like actin could induce an adverse autoimmune response (Allison, 1977).

In conclusion, we would like to emphasize the importance of antigen presentation in vaccination studies. Our studies suggest at least one simple method to analyse a surface glycoprotein preparation before its use in immunogenicity studies. This can be done by sedimentation velocity analysis in the preparative or the analytical ultracentrifuge. Preparations can in this way be standardized and the problem of multimers versus monomers properly addressed.

We wish to thank Ulla Hallen, Patricia Marinello and Hilkka Virta for expert technical assistance and Karl Matlin for a critical reading of the manuscript.

## REFERENCES

ALLISON, A. C. (1977). Autoimmunity, genetic, immunologic, virologic and clinical aspects. In *Autoimmune Diseases – Concepts of Pathogenesis and Control*, pp. 92–139. Edited by N. Talal. New York & London: Academic Press.

BACHMAYER, H., SCHMIDT, G. & LIEHL, E. (1976). Preparation and properties of a novel influenza subunit vaccine. *Post-Graduate Medical Journal* **52**, 360–367.

BALCAROVA, J., HELENIUS, A. & SIMONS, K. (1981). Antibody response to spike protein vaccines prepared from Semliki Forest virus. *Journal of General Virology* **53**, 85–92.

BLIKSTAD, J. F., MARKEY, F., CARLSSON, L., PERSSON, T. & LINDBERG, U. (1978). Selective assay of monomeric and filamentous actin in cell extract using inhibition of deoxyribonuclease I. *Cell* **15**, 935–943.

BRADY, M. I. & FURMINGER, I. G. (1976). A surface antigen influenza vaccine. 2. Pyrogenicity and antigenicity. *Journal of Hygiene* **77**, 173–180.

CHOPPIN, P. W. & COMPANS, R. W. (1975). Reproduction of paramyxoviruses. In *Comprehensive Virology*, vol. 4, pp. 99–178. Edited by H. Fraenkel-Conrat & R. R. Wagner. New York: Plenum Press.

COX, J. H., DIETZCHOLD, B. & SCHNEIDER, L. G. (1977). Rabies virus glycoprotein. II. Biological and serological characterization. *Infection and Immunity* **16**, 743–759.

EMTAGE, J. S. & CAREY, N. H. (1980). The production of vaccines by recombinant DNA techniques. In *New Developments with Human and Veterinary Vaccines*, pp. 367–375. Edited by A. Mizrahi, I. Hertman, M. A. Klingberg & A. Hohn. New York: Alan R. Liss.

GERSHON, R. K. (1974). T cell control of antibody production. In *Contemporary Topics in Immunology*, vol. 3, pp. 1–40. Edited by M. D. Cooper & N. L. Warner. New York: Plenum Press.

GINSBERG, H. S. (1975). Subunit viral vaccines. In *Viral Immunology and Immunopathology*, pp. 317–326. Edited by A. L. Notkins. New York: Academic Press.

GROSS, P. A., ENNIS, F. A., GAERLAN, P. F., DENSON, L. J., DENNING, C. R. & SCHIFFMAN, D. (1977). A controlled doubleblind comparison of reactogenicity, immunogenicity and protective efficacy of whole-virus and split-product influenza vaccines in children. *Journal of Infectious Diseases* **36**, 623–632.

HEINZ, F. X. & KUNZ, CH. (1980). Formation of polymeric glycoprotein complexes from a flavivirus: a tick-borne encephalitis virus. *Journal of General Virology* **49**, 125–132.

HELENIUS, A. & SIMONS, K. (1975). Solubilization of membranes by detergents. *Biochimica et biophysica acta* **415**, 29–79.

HELENIUS, A. & VON BONSDORFF, C. (1976). Semliki Forest virus membrane proteins. Preparation and characterization of spike complexes soluble in detergent-free medium. *Biochimica et biophysica acta* **436**, 895–899.

HILLEMAN, M. R. (1976). Herpes simplex vaccines. *Cancer Research* **36**, 857–858.

HOMMERS, G., GILKENS, A. & SCHAAKE, J. (1982). An indirect enzyme-linked immunoabsorbent assay for the detection of antibodies to visna viruses. *Veterinary Microbiology* (in press).

HUNSMANN, G., SCHNEIDER, J. & SCHULZ, A. (1981). Immunoprevention of Friend virus-induced erythroleukemia vaccination with viral envelope glycoprotein complexes. *Virology* **113**, 602–612.

KLEID, D. G., YANSURA, D., SMALL, B., DOWBENKA, D., MOORE, D. M., GRUBMAN, M. J., MCKERCHER, P. D., MORGAN, D. O., ROBERTSON, B. H. & BACHRACH, H. L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: response in cattle and swine. *Science* **214**, 1125–1129.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LAVER, G. W. & VALENTINE, R. C. (1969). Morphology of the isolated hemagglutinin and neuraminidase subunits of influenza virus. *Virology* **38**, 105–119.

LERNER, R. A., GREEN, N., ALEXANDER, H., LIU, F. T., SUTCLIFFE, J. G. & SHINNICK, J. M. (1981). Chemically synthesised peptides predicted from the nucleotide sequence of the hepatitis virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proceedings of the National Academy of Sciences, U.S.A.* **78**, 3403–3407.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

LUUKKONEN, A., GAHMBERG, C. G. & RENKONEN, O. (1977). Surface labeling of Semliki Forest virus glycoproteins using galactose oxidase. *Virology* **76**, 55–59.

MCLEAN, B., SONZA, S. & HOLMES, I. H. (1980). Measurement of immunoglobulin A, G and M class rotavirus antibodies in serum and mucosal secretions. *Journal of Clinical Microbiology* **12**, 314–319.

MARTIN, R. G. & AMES, B. N. (1961). A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *Journal of Biological Chemistry* **236**, 1372–1379.

MATLIN, K., REGGIO, H., HELENIUS, A. & SIMONS, K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. *Journal of Cell Biology* **91**, 601–613.

MATLIN, K., REGGIO, H., HELENIUS, A. & SIMONS, K. (1982). Pathway of vesicular stomatitis virus entry leading to infection. *Journal of Molecular Biology* **156**, 609–631.

MOREIN, B., HELENIUS, A., SIMONS, K., PETTERSSON, R., KÄÄRIÄINEN, L. & SCHIRRMACHER, V. (1978). Effective subunit vaccines against an enveloped animal virus. *Nature, London* **276**, 715–718.

MOREIN, B., SUNDQUIST, B., HÖGLUND, S., HELENIUS, A. & SIMONS, K. (1981). Protein micelles and virosomes from the surface glycoproteins of parainfluenza 3 virus. *Protides of the Biological Fluids* **29**, 101–104.

MULLER, G. M., SHAPIRA, M. & ARNON, R. (1982). Anti-influenza response achieved by immunization with a synthetic conjugate. *Proceedings of the National Academy of Sciences, U.S.A.* **79**, 569–573.

SCHEID, A., CALIGUIRI, L. A., COMPANS, R. W. & CHOPPIN, P. W. (1972). Isolation of paramyxovirus glycoproteins. *Virology* **50**, 640–652.

SCHNEIDER, J., FALK, H. & HUNSMANN, G. (1980). Envelope polypeptides of Friend leukemia virus: purification and structural analysis. *Journal of Virology* **33**, 597–605.

SHIBUTA, H., KANDA, T., ADACHI, A. & YOGO, Y. (1979). Characterization of bovine parainfluenza virus type 3. *Microbiology and Immunology* **23**, 617–628.

SIMONS, K., HELENIUS, A., LEONARD, K., SARVAS, M. & GETHING, M. J. (1978). Formation of protein micelles from amphiphilic membrane proteins. *Proceedings of the National Academy of Sciences, U.S.A.* **75**, 5306–5310.

SIMONS, K., HELENIUS, A., MOREIN, B., BALCAROVA, J. & SHARP, M. (1980). Development of effective subunit vaccines against enveloped viruses. In *New Developments with Human and Veterinary Vaccines*, pp. 217–228. Edited by A. Mizrahi, I. Hertman, M. A. Klingberg & A. Hohn. New York: Alan R. Liss.

SKELLY, J., HOWARD, C. R. & ZUCKERMAN, A. J. (1981). Hepatitis B polypeptide vaccine in micelle form. *Nature, London* **280**, 51–54.

TADA, T. & OKUMURA, K. (1979). The role of antigen-specific T cell factors in the immune response. *Advances in Immunology* **28**, 28–67.

WALLER, T., LYNGSET, A., ELVANDER, M. & MOREIN, B. (1980). Immunological diagnosis of encephalitozoonosis from post-mortem specimens. *Veterinary Immunology and Immunopathology* **1**, 353–360.

WELLS, P. W., SHARP, J. M., BURRELS, L., RUSHTON, B. & SMITH, W. D. (1976). The assessment in sheep of an inactivated vaccine of parainfluenza 3 virus incorporating double stranded RNA as adjuvant. *Journal of Hygiene* **77**, 255–261.

WELLS, P. W., SHARP, J. M., RUSHTON, B., GILMOUR, M. J. L. & THOMPSON, D. A. (1978). The effect of vaccination with a parainfluenza type 3 virus on pneumonia resulting from infection with parainfluenza type 3 virus and *Pasteurella haemolytica*. *Journal of Comparative Pathology* **88**, 253–259.

(*Received 6 December 1982*)

1: J Infect Dis. 1985 Dec;152(6):1219-30.



# Glycoproteins of human parainfluenza virus type 3: characterization and evaluation as a subunit vaccine.

**Ray R, Brown VE, Compans RW.**

The envelope glycoproteins of human parainfluenza type 3 virus were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reactivity with specific monoclonal antibodies. The molecular weight of the hemagglutinin-neuraminidase (HN) glycoprotein was found to be 72,000, and the fusion (F) glycoprotein appeared to consist of 74,000 (F0) or 56,000 (F1) species. Envelope glycoproteins were solubilized with octyl-glucoside and, after removal of the detergent by dialysis, were used for immunization of hamsters. Other animals were immunized with a formalin-inactivated preparation of whole virus. A single subcutaneous immunization with these antigen preparations induced a serum antibody response to the HN and F glycoproteins, as determined by plaque neutralization, hemagglutination inhibition, inhibition of virus-induced cell fusion, and immune precipitation tests. An IgG antibody response to both glycoproteins was also observed in bronchial washings. Animals immunized with the highest dose of envelope glycoproteins showed complete protection from challenge infection, whereas immunization with inactivated virus did not completely protect animals.

PMID: 2999259 [PubMed - indexed for MEDLINE]

JOURNAL OF VIROLOGY, Nov. 1987, p. 3416–3423
0022-538X/87/113416-08$02.00/0

Vol. 61, No. 11

# Expression of the F and HN Glycoproteins of Human Parainfluenza Virus Type 3 by Recombinant Vaccinia Viruses: Contributions of the Individual Proteins to Host Immunity

MELANIE K. SPRIGGS,[1] BRIAN R. MURPHY,[1] GREGORY A. PRINCE,[2] ROBERT A. OLMSTED,[1] AND PETER L. COLLINS[1]*

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892,[1] and Department of International Medicine, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21205[2]*

Received 27 March 1987/Accepted 13 July 1987

**cDNA clones containing the complete coding sequences for the human parainfluenza virus type 3 (PIV3) fusion (F) and hemagglutinin-neuraminidase (HN) glycoprotein genes were inserted into the thymidine kinase gene of vaccinia virus (WR strain) under the control of the P7.5 early-late vaccinia virus promotor. The recombinant vaccinia viruses, designated vaccinia-F and vaccinia-HN, expressed glycoproteins in cell culture that appeared to be authentic with respect to glycosylation, disulfide linkage, electrophoretic mobility, cell surface expression, and, in the case of the HN protein, biological activity. Cotton rats inoculated intradermally with vaccinia-HN developed serum neutralizing antibody titers equal to that induced by respiratory tract infection with PIV3, whereas animals receiving vaccinia-F had threefold lower neutralizing antibody titers. A single immunization with either recombinant vaccinia virus induced nearly complete resistance in the lower respiratory tract of these animals. With regard to protection in the upper respiratory tract, animals immunized with vaccinia-HN or vaccinia-F exhibited reductions in PIV3 replication of >3,000-fold and 6-fold, respectively. This large difference (>500-fold) in reduction of PIV3 replication in the upper respiratory tract was in contrast to the relatively modest difference (3-fold) in serum neutralizing antibody titers induced by vaccinia-HN versus vaccinia-F. This dissociation between the level of neutralizing antibodies and protection suggested that immunity to PIV3 is complex, and that immune mechanisms other than serum neutralizing antibodies make important contributions to resistance to infection. Overall, under these experimental conditions, vaccinia-HN induced a substantially more protective immune response than did vaccinia-F.**

Human parainfluenza virus type 3 (PIV3), a member of the family *Paramyxoviridae*, is second only to respiratory syncytial virus (RSV) as a primary cause of severe lower respiratory tract disease in children under 1 year of age (4). Older children and adults are frequently reinfected, resulting in less severe upper respiratory tract (URT) illness. The virus occurs worldwide, and virtually all children are infected by age 4 (4).

Attempts to develop an effective PIV3 vaccine have been largely unsuccessful. Field evaluations of killed PIV3 vaccines demonstrated an increase in virus-specific serum antibodies in vaccinees without significant protection against disease (11, 28, 44). The primary immunogens of PIV3 are its two surface glycoproteins. The F protein, ($M_r$ 60,000 to 63,000), mediates viral penetration, hemolysis, and syncytium formation, which is a characteristic cytopathic effect and may be important in cell-cell spread (5, 13, 21, 33, 43, 46). The F protein is synthesized as an inactive precursor $F_0$, which is activated by proteolytic cleavage into two disulfide-linked subunits, $F_1$ and $F_2$ (12, 25, 36). The second surface glycoprotein, HN ($M_r$ 69,000 to 72,000), is responsible for viral attachment and exhibits both hemagglutination and neuraminidase activities (13, 33, 35, 43, 46). Ray et al. have shown that subcutaneous inoculation of hamsters with solubilized envelope glycoproteins can protect their l wer respiratory tract against challenge with PIV3 (32). However, immunization with the individual glycoproteins has not been described, and the relative contributions of the individual glycoproteins in eliciting a protective immune response was unknown.

The development of techniques for producing live vaccinia virus (VV) recombinants expressing foreign genes has provided a novel method for studying immune responses to individual proteins (19). Several viral surface antigens, including those of the pneumovirus RSV, have been successfully expressed by VV recombinants and shown to elicit protective neutralizing antibodies in experimental animals (9, 27). Recently, the nucleotide sequences of the PIV3 F and HN mRNAs were determined from virion RNA and cDNA clones (8, 41). We have constructed cDNAs which contain the complete coding regions of the F and HN protein genes under the control of the early-late P7.5 VV promoter, and we have inserted each of these genes into the thymidine kinase (TK) gene of the WR strain of VV. The resulting recombinants, designated vaccinia-F and vaccinia-HN, were then used to inoculate cotton rats, an experimental animal permissive for PIV3 infecti n. In this paper, we describe (i) the expression of the F and HN glycoproteins by VV recombinants in cell culture, (ii) the development of serum neutralizing antibodies to both F and HN glycoproteins by cotton rats inoculated with vaccinia-F or vaccinia-HN, (iii) the induction by either VV recombinant of resistance to viral replication in the lower respiratory tract (LRT) f cotton rats

* Corresponding author.

challenged with PIV3, and (iv) a 500-fold greater restriction of PIV3 replication in the URT of animals previously immunized with vaccinia-HN compared with those immunized with vaccinia-F, despite the development of only a 3-fold greater neutralizing antibody titer by the vaccinia-HN group.

## MATERIALS AND METHODS

**Viruses and cells.** Purified stocks of VV (WR strain) and recombinant VV were prepared from infected CV-1 monolayers as described previously (9). PIV3 strain 47885 was plaque purified three times and propagated in HEp-2 cells. Assays for biological activity of expressed proteins were performed in HEp-2 cells. Recombinant VV vSC-14 (3) c ntains the hepatitis B virus surface antigen gene under the control of the P7.5 promoter and was used as a negative control.

**Construction of VV recombinants.** Two previously described overlapping F cDNA clones were cut at a unique *Bgl*I site and ligated to form a complete cDNA of the F mRNA (41). To remove noncoding sequences and homopolymer tails, F and HN cDNAs were inserted into the *Pst*I sites of pTZ18R (Pharmacia Fine Chemicals, Piscataway, N.J.) and the replicative form of M13 mp18, respectively. A single-stranded template for mutagenesis was prepared according to the supplier's protocols for pTZ18R and conventional methods for M13. Oligonucleotide-directed mutagenesis (49) was utilized to introduce *Bam*HI sites within 3 to 6 nucleotides of the translational initiation and termination sites. The oligonucleotides (which were antisense) were as follows, identified according to gene and gene end, with the *Bam*HI sites and termination or initiation codons in boldface type: F-3', 5'TAGAGTCGACCTGCAGGATCCCTGTCATTTGTTTGTTAAT; F-5', 5'TATTGAGGTTGGCATGATGGATCCATGCAAGCTTGGCACT; HN-3', 5'GTGCCAAGCTTGCATGGATCCTTATGATTAACTGCAGCTTGG; HN-5', 5'CCAGTATTCCATCTCGGATCCCCGGGTACCGAGCTCG.

After the mutagenesis, the sequences of the HN and F cDNAs were confirmed in their entirety by dideoxynucleotide sequencing with synthetic oligonucleotides as primers (48).

**C nstruction of the VV recombinants.** The resected F and HN cDNAs were isolated by digestion with *Bam*HI, made blunt ended with T4 DNA polymerase, and inserted into the *Sma*I site of the VV plasmid coexpression vector pSC11 (3). Features of this construct include the following: (i) the foreign cDNA is under the control of the early-late VV promoter P7.5; (ii) the vector contains the *Escherichia coli* β-galactosidase gene under the control of the late VV promoter P11; and (iii) both chimeric genes are flanked by VV TK gene sequences. Upon transfection of VV-infected cells, the TK gene sequences direct homologous recombination into the TK locus of the VV genome, yielding live recombinant VV that are identified colorimetrically by the presence of β-galactosidase activity. Methods for obtaining such recombinants have been described in detail (3, 9). The mRNAs expressed from the chimeric 7.5K-F and 7.5K-HN genes would each contain a 5' noncoding sequence of 52 nucleotides consisting of 44 nucleotides representing the start of the VV 7.5K mRNA, 5 nucleotides representing the filled-in *Bam*HI site, and the original 3 PIV3-specific nucleotides preceding the PIV3 translational start codon (3, 9, 38). In comparison, F and HN mRNAs expressed by PIV3 contain 5' noncoding regions of 193 and 73 nucleotides, respectively (8, 40, 41).

**Radiolabeling and immunoprecipitation f PIV3-infected cell lysates.** CV-1 cells were infected with VV recombinants (30 PFU per cell) for 2 h and then incubated for 4 to 6 h with 20 μCi of [$^{35}$S]methionine per ml or 16 h with 50 μCi of [$^{3}$H]glucosamine per ml. Similar conditions were used to label cells infected with PIV3 (5 PFU per c ll), except that the labeling period for [$^{35}$S]methionine was 24 to 28 h postinfection. Infected cell lysates were prepared as described previously (26), and immunoprecipitations were carried out with anti-PIV3 hyperimmune cotton rat sera and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). Samples were boiled in electrophoresis sample buffer with or without 2-mercaptoethanol and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18).

**Indirect immunofluorescence.** CV-1 cells were grown on glass cover slips and infected with 1 PFU of PIV3 or a VV recombinant per cell. Cells were incubated for 24 h and fixed with 3% (vol/vol) Formalin in phosphate-buffered saline for cell surface immunofluorescence. Fixed cells were reacted sequentially with anti-PIV3 hyperimmune cotton rat serum, rabbit anti-cotton rat serum, and goat anti-rabbit immunoglobulin G fluorescein-conjugated antibody (Cooper Biomedical, Inc., West Chester, Pa.) (27).

**Immunological response to immunization and protective efficacy.** Young adult cotton rats (*Sigmodon fulviventer*) were immunized by intradermal (i.d.) inoculation with one or both VV recombinants ($10^{8.0}$ PFU each) or by intranasal (i.n.) inoculation with PIV3 ($10^{6.0}$ PFU); 28 days after immunization, animals were bled and then challenged with PIV3 i.n. ($10^{5.0}$ PFU). Animals were sacrificed 4 days after challenge, at the peak of PIV3 replication in the respiratory tract (unpublished observation). PIV3 specific antibody responses were measured by an enzyme-linked immunosorbent-assay (ELISA) with purified PIV3 as the antigen (31). Hemagglutination inhibition assays were performed as described previously (6). PIV3 serum neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction neutralization assay. PIV3 titers in the nasal turbinates and lungs were determined by 50% tissue culture-infective dose ($TCID_{50}$) assays. Each assay was done in MK-2 cells and included two wells per dilution. The minimum level of detectability was determined by using serial 10-fold dilutions of homogenates in a 500-μl inoculation volume. Detectable virus in a single well receiving the lowest ($10^{0}$) dilution of a 10% (wt/vol) homogenate permitted a minimum level of detectability of $10^{1.3}$ $TCID_{50}$ per g of tissue. No detectable virus in either well of the lowest dilution of homogenate represents a value $\leq 10^{0.8}$ $TCID_{50}$ per g of tissue.

## RESULTS

**Expression of the F and HN glycoproteins by VV recombinants.** To examine the intracellular expression of PIV3 glycoproteins by the VV recombinants, CV-1 cells were infected with vaccinia-F or vaccinia-HN, and radiolabeled cell lysates were prepared and analyzed by immunoprecipitation with anti-PIV3 hyperimmune cotton rat serum (Fig. 1).

Polyacrylamide gel electrophoresis under reducing conditions (Fig. 1, lanes d through h) identified the $F_0$ precursor ($M_r$ ~60,000) and the $F_1$ subunit ($M_r$ ~51,000), but did not resolve the $F_2$ subunit ($M_r$ ~9,000). Under n nreducing conditions (Fig. 1, lanes a through c), the F protein expressed by both vaccinia-F and PIV3 migrated as two closely

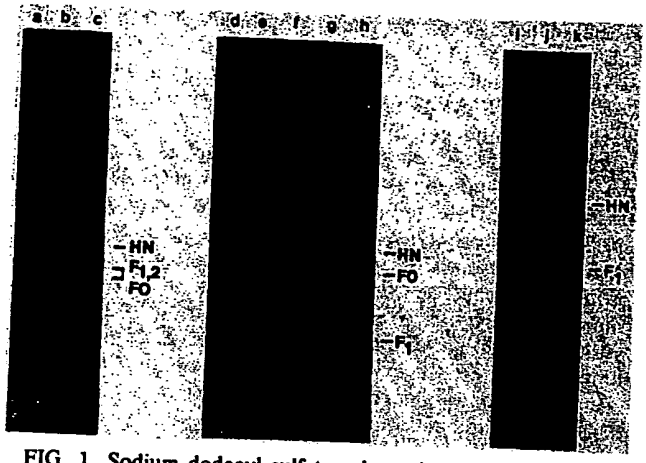



FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated proteins synthesized by vaccinia-F, vaccinia-HN, or PIV3. CV-1 cells were infected with recombinant VVs or PIV3 and incubated with [$^{35}$S]methionine or [$^{3}$H]glucosamine (see Materials and Methods). All lysates were clarified and incubated with anti-PIV3 hyperimmune cotton rat (αPIV3) serum. Immunoprecipitates were analyzed by 10% polyacrylamide gel electrophoresis under nonreducing (lanes a through c) or reducing (lanes d through k) conditions. Lanes: a and d, $^{35}$S-labeled proteins from VV vSC-14-infected cells; b and e, $^{35}$S-labeled proteins from PIV3-infected cells; c and f, $^{35}$S-labeled proteins from vaccinia-F-infected cells; g, [$^{3}$H]glucosamine-labeled proteins from vaccinia-F-infected cells; h and j, [$^{3}$H]glucosamine-labeled proteins from VV vSC-14-infected cells; i [$^{3}$H]glucosamine-labeled proteins from vaccinia-HN-infected cells; k, [$^{3}$H]glucosamine-labeled proteins from PIV3-infected cells.

spaced bands ($M_r$ ~60,000 to 63,000). These were interpreted as representing $F_0$ and one or more electrophoretic species of disulfide-linked $F_1$ and $F_2$ (designated $F_{1,2}$), although the bands were not identified individually. This indirectly confirmed the presence of the $F_2$ subunit and showed that the F protein expressed by vaccinia-F was very similar to that of PIV3 on the basis of both proteolytic processing and disulfide linkage. Furthermore, the $F_0$ and $F_1$ proteins expressed by vaccinia-F were glycosylated (Fig. 1, lane g) and on this basis were indistinguishable from the $F_0$ and $F_1$ proteins expressed by PIV3 (data not shown).

The HN proteins synthesized by vaccinia-HN (Fig. 1, lane i) and PIV3 (lane k) were indistinguishable by electrophoretic mobility under reducing (Fig. 1) and nonreducing (data not shown) conditions. Consistent with previous reports (43, 46), nonreduced HN protein migrated as a monomer with a slightly increased electrophoretic mobility, indicative of intramolecular disulfide bonds. The HN protein expressed by vaccinia-HN and PIV3 shared this feature of secondary structure. This is in contrast to the HN proteins of other paramyxoviruses such as Sendai virus and Newcastle disease virus, which exist as disulfide-linked multimers under nonreducing conditions (20, 39).

The HN proteins expressed by vaccinia-HN-infected cells were assayed for biological activity by incubating infected HEp-2 monolayers with guinea pig erythrocytes at 4°C. C lls infected with vaccinia-HN (1 PFU per cell) exhibited extensive hemadsorption when assayed at 16 h postinfection (Fig. 2B), whereas monolayers infected in parallel with the parental VV showed no hemadsorption (Fig. 2A). The neuraminidase activity of the expressed HN proteins was demonstrated indirectly by the elution of bound erythrocytes when vaccinia-HN-infected, hemadsorbed monolayers were incubated at 37°C for 30 min (Fig. 2C). Elution of erythrocytes at 37°C was presumed to be due to the neuraminidase activity of the expressed HN protein, although we cannot exclude the possibility that the cells contained an endogenous neuraminidase activity.

The expression of F and HN glycoproteins at the surface of recombinant infected cells was examined by indirect immunofluorescent staining of nonpermeabilized (Formalin-fixed) cells (Fig. 3). The level of expression of PIV3 antigen in cells infected with vaccinia-F (Fig. 3B) and vaccinia-HN (Fig. 3C) was equal to or exceeded that of PIV3 infected cells (Fig. 3A). Under these conditions, syncytium formation specific to vaccinia-F was not clearly demonstrated because of the rapid cytopathic effect of VV in CV-1 cells, consistent with previous results (27).

**Serologic response to infection by vaccinia-F and vaccinia-HN.** Cotton rats were inoculated i.d. with vaccinia-F, vaccinia-HN, or both to determine the level and nature of serum antibodies induced by the individual glycoproteins. In cotton rats, these conditions of inoculation did not result in dermal lesions with either the parental strain WR VV or the recombinant VVs. However, such inoculations induced high titers of VV-specific serum antibodies (R. A. Olmsted, B. R.

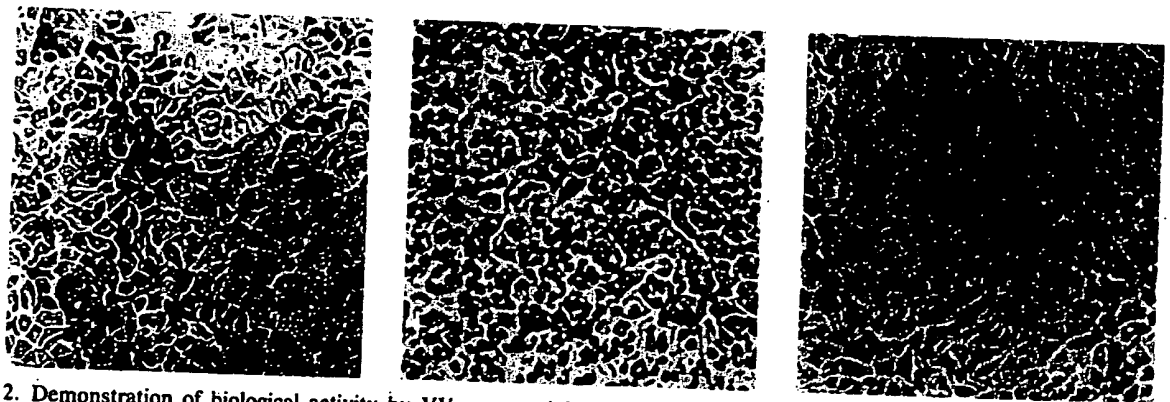


FIG. 2. Demonstration of biological activity by VV-expressed HN protein. HEp-2 monolayers were infected (1 PFU per cell) with vaccinia-HN (B and C) or parental WR VV (A) and incubated at 4°C with guinea pig erythrocytes 16 h postinfection. After photography, the hemadsorbed monolayers were incubated at 37°C for 30 min to demonstrate elution of the erythrocytes (C), suggesting that the expressed HN protein possessed a functional neuraminidase.

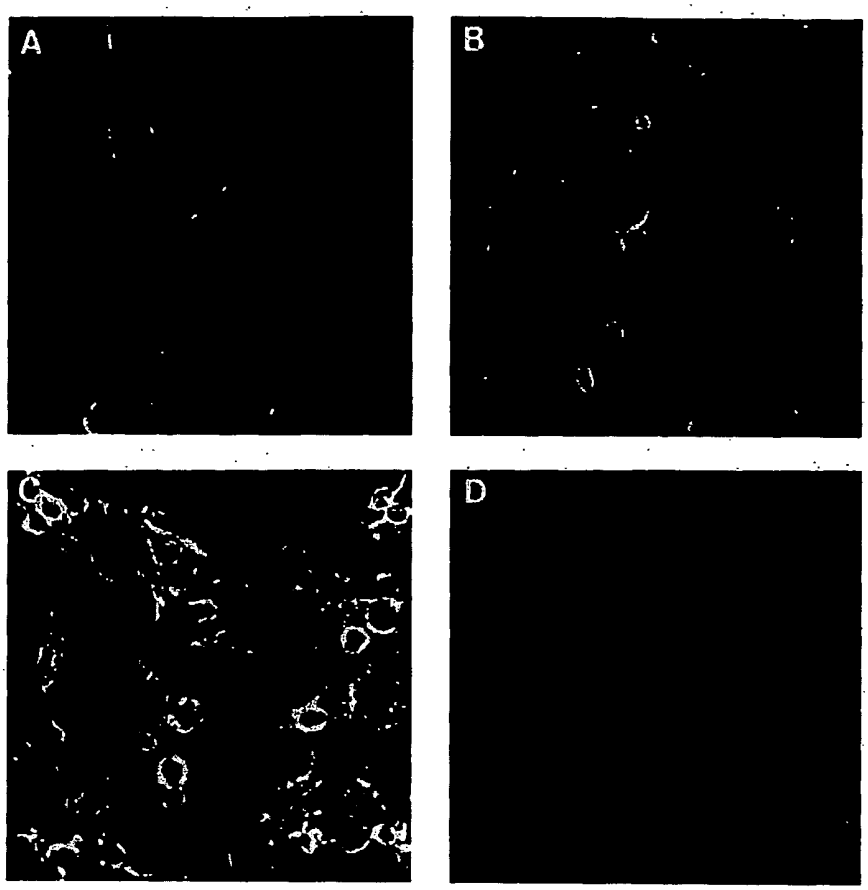



FIG. 3. Indirect cell surface immunofluorescence of VV recombinant infected cells. CV-1 cells were grown on glass cover slips and infected (1 PFU per cell) with PIV3 (A), vaccinia-F (B), vaccinia-HN (C), or VV vSC-14 (D). At 24 h postinfection, cells were fixed in 3% Formalin in phosphate-buffered saline for 15 min at room temperature. Fixed cells were incubated for 30 min at 37°C with anti-PIV3 hyperimmune cotton rat serum, washed, incubated for 30 min at 37° with a rabbit anti-cotton rat serum, washed, and incubated for 30 min with a goat anti-rabbit immunoglobulin G fluorescein-conjugated antibody.

Murphy, P. L. Collins, M. Buller, and B. Moss, unpublished data) and high titers of serum antibodies specific to the expressed foreign protein (27) (Table 1), indicating that an active VV infection had followed the inoculation. For comparison, other animals were inoculated i.n. with PIV3, which results in a self-limiting, asymptomatic respiratory tract infection that confers complete protection against reinfection (G. A. Prince, manuscript in preparation). Animals were bled 28 days postinoculation, and the sera were assayed for PIV3 antibodies by plaque reduction, hemagglutination inhibition (HI) assay, and ELISA with purified virus as the antigen (Table 1).

TABLE 1. Serum antibody response of cotton rats to PIV3 infection or recombinant VV inoculation[a]

| Immunization (route) | Dose (PFU) | No. of animals | Reciprocal geometric mean serum antibody titer | | |
|---|---|---|---|---|---|
| | | | ELISA[b] | Neutralization[c] | HI[d] |
| PIV3 (i.n.) | $10^6$ | 18 | 7,525 | 1,326 | 145 |
| Vaccinia-F (i.d.) plus vaccinia-HN (i.d.) | $10^8$, $10^8$ | 17 | 8,018 | 1,069 | 67 |
| Vaccinia-HN (i.d.) | $10^8$ | 15 | 4,889 | 1,391 | 122 |
| Vaccinia-F (i.d.) | $10^8$ | 15 | 1,940 | 459 | ≤2 |
| VV vSC-14 (i.d.)[e] | $10^8$ | 17 | 501 | <11 | ≤2 |

[a] Serum was collected from all groups 28 days postimmunization.
[b] Purified PIV3 virions were used as the solid-phase antigen. The ELISA titer in VV vSC-14 control group was concluded to be nonspecific binding of VV-induced antibodies (see text). The mean preimmunization ELISA titer of control group was 1:127.
[c] Measured by 60% plaque reduction neutralization assay.
[d] Dilution that inhibits erythrocyte agglutination by four agglutinating units of virus.
[e] VV recombinant expressing hepatitis B virus surface antigen.

Sera from animals receiving both VV recombinants exhibited an ELISA titer equal to that of sera from PIV3-infected animals, whereas animals that received vaccinia-HN or vaccinia-F alone had titers that were 1.5- and 4-fold lower, respectively. Because the antigen for ELISA was whole virus, the titer of sera from PIV3-infected animals would represent reactivity with multiple viral antigens. Animals which received the VV control vSC-14 also exhibited a slight rise in PIV3-reactive ELISA titer when compared with preimmunization sera of this group (Table 1). This response might be due to nonspecific immune stimulation by VV or to a low level of cross-reactivity by VV-specific antibodies.

Infection with vaccinia-HN induced levels of PIV3 specific serum neutralizing antibodies that were equal to that induced by infection with PIV3. In contrast, vaccinia-F infection induced threefold lower neutralizing antibodies than did vaccinia-HN or PIV3 infection. Animals which received both vaccinia-F and vaccinia-HN had titers of serum neutralizing antibodies that were not significantly

TABLE 2. Response of cotton rats to PIV3 challenge[a]

| Immunization (route) | Dose (PFU) | No. of animals | Recovery of virus from respiratory tract tissues | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Nasal turbinates | | | Lungs | | |
| | | | % With detectable virus | Mean titer ± SEM ($log_{10}$ $TCID_{50}$/g)[b] | Reduction in titer ($log_{10}$)[c] | % With detectable virus | Mean titer ± SEM ($log_{10}$ $TCID_{50}$/g)[b] | Reduction in titer ($log_{10}$)[c] |
| PIV3 (i.n.) | $10^6$ | 12 | 0 | $\leq 0.8 \pm 0.0$[d] | $\geq 3.9$ | 0 | $\leq 0.8 \pm 0.0$[d] | $\geq 3.4$ |
| Vaccinia-HN (i.d.) | $10^8$ | 11 | 36 | $1.2 \pm 0.2$ | 3.5 | 9 | $1.0 \pm 0.2$[d] | 3.2 |
| Vaccinia-F (i.d.) | $10^8$ | 11 | 100 | $3.9 \pm 0.2$[e] | 0.8 | 18 | $0.9 \pm 0.1$[d] | 3.3 |
| VV vSC-14 (i.d.) | $10^8$ | 11 | 100 | $4.7 \pm 0.2$ | | 100 | $4.2 \pm 0.2$ | |

[a] Animals were challenged 28 days postimmunization with $10^{5.0}$ PFU of PIV3 i.n. Animals were sacrificed 4 days later.
[b] Minimum level of detectability was $10^{1.3}$ $TCID_{50}$/g (see Materials and Methods).
[c] Compard with VV vSC-14 control group.
[d] $P < 0.001$ compared with VV vSC-14 control group by Student's $t$ test.
[e] $P < 0.02$ compared with VV vSC-14 control group by Student's $t$ test.

different from animals receiving vaccinia-HN alone or PIV3 by i.n. infection.

The glycoprotein specificity of the serum antibodies also was examined for the ability to inhibit hemagglutination by purified PIV3. Inoculation with vaccinia-HN induced HI titers which were similar to those induced by PIV3 infection (Table 1). Vaccinia-F or vSC-14-specific sera exhibited essentially no detectable HI activity. Sera from animals receiving both recombinants exhibited an HI titer that was twofold lower than that seen for sera from PIV3- or vaccinia-HN-infected animals, perhaps reflecting a slightly decreased immune response to the HN protein when the VV recombinants were administered together.

**Protective efficacy of vaccinia-F and vaccinia-HN.** To test the ability of the VV recombinants to confer protective immunity, the groups of cotton rats described above were challenged with $10^{5.0}$ PFU of PIV3 i.n. 28 days postimmunization. Four days later, animals from each group were randomly chosen and sacrificed, and the levels of virus replication in the nose and lungs were measured (Table 2). Control animals inoculated with vSC-14 (a negative control) supported the replication of 4.2 $log_{10}$ units of virus per g of lung tissue and 4.7 $log_{10}$ units of virus per g of nasal turbinate (Table 2), levels comparable to those of unvaccinated animals (data not shown). Animals vaccinated i.n. with PIV3 showed no detectable virus growth in either the LRT or URT. Animals receiving vaccinia-HN showed nearly complete protection of the LRT and greatly reduced (3,000-fold) virus replication in the URT. Animals receiving vaccinia-F also showed nearly complete protection of the LRT, but had less protection (sixfold reduction in virus titer) of the URT. The level of PIV3 replication in cotton rats receiving both vaccinia-F and vaccinia-HN recombinants was similar to that in animals which received vaccinia-HN alone (data not shown). Thus, under the conditions of this study, the protection conferred by vaccinia-HN exceeded that conferred by vaccinia-F.

## DISCUSSION

Recombinant VV were constructed which carry the complete coding sequences for either the PIV3 F or HN gene under the control of the early-late P7.5 VV promoter. These recombinants expressed HN and F proteins in cell culture which appeared to be authentic with resp ct to electrophoretic mobility, proteolytic processing, glycosylation, antigenic specificity, and, in the case of the HN protein, biological activity. The proteins were expressed at the surface of recombinant-infected cells, as well as intracellularly, in an abundance equal to or greater than that found in PIV3-infected cells.

Immunization of cotton rats with the VV recombinants established that each of the two PIV3 glycoproteins administered individually can induce a vigorous immune response that provides substantial protection against PIV3 infection. A single i.d. inoculation of cotton rats with vaccinia-F or vaccinia-HN induced high levels of PIV3-specific serum antibodies. Vaccinia-HN was found to stimulate a 2.5-fold higher titer of PIV3-specific serum antibodies (as measured by ELISA) and a 3-fold higher neutralizing antibody titer (as measured by 60% plaque reduction of PIV3) than did vaccinia-F. The LRTs of both groups of cotton rats were almost completely protected against challenge with PIV3. The URTs of immunized cotton rats, however, differed in the level of immunity induced by the two recombinant viruses.

This incomplete protection in the URT permitted a comparison of the relative abilities of vaccinia-HN and vaccinia-F to restrict replication of PIV3 in vivo. Animals receiving vaccinia-F exhibited a 6-fold reduction of virus growth in the URT compared with control animals, whereas the URT of the vaccinia-HN group exhibited a greater than 3,000-fold reduction in detectable virus. Thus, although vaccinia-HN induced only a 3-fold greater level of serum neutralizing antibodies than did vaccinia-F, it conferred a 500-fold greater level of protection in the URT. This suggested that immune mechanisms in addition to serum neutralizing antibodies were involved in the protective immunity. These additional mechanisms could include cellular immune responses as well as the stimulation of antibodies that are protective in vivo but do not neutralize viral infectivity in vitro, as have been described for influenza A and Sindbis viruses (16, 23, 37, 42). The observation that neither VV recombinant conferred complete protection in the URT was not unexpected because the i.d. route of administration presumably would not have stimulated local immune responses in the respiratory tract (29, 30).

Studies of influenza virus infections in humans have shown that a direct correlation exists between the level of virus replication in the URT and manifestation of clinical symptoms. Although this correlation has not been shown conclusively for PIV3, by analogy with influenza virus a reduction of 3,000-fold in virus replication in the URT as observed in animals immunized with vaccinia-HN would be expected to significantly diminish illness. Additionally, for RSV, serious disease is associated with viral replication in the LRT, and a similar situation probably exists for PIV3.

The high level of protection of the LRT conferred by both the VV rec mbinants would be expected to block serious viral disease, whereas the level of protection of the URT afforded by vaccinia-HN would permit reduced levels of PIV3 replication, thereby boosting immunity in the respiratory tract without the development of serious illness.

Recently, the expression and protective efficacy of VV recombinants containing the fusion (F) glycoprotein and putative attachment glycoprotein (G) of RSV have been described for the cotton rat model system (9, 27). The RSV F protein is analogous in structure and function to the PIV3 F protein, and the two proteins share moderate amino acid sequence homology (41). The RSV G protein is thought to be the attachment protein and therefore would be analogous to the PIV3 HN protein, but it lacks hemagglutinin and neuraminidase activities and does not share amino acid sequence homology with the PIV3 HN protein. In the cotton rat system, RSV vaccinia-F elicited a sixfold higher titer of neutralizing antibodies than did RSV vaccinia-G and provided greater resistance to replication in both the lungs and noses of immunized animals (27). Thus, for RSV, the F protein appeared to be significantly more immunogenic and protective than the G protein. In contrast, in the present study, recombinant VV expressing the HN protein, the PIV3 counterpart to the G protein, induced a moderately higher level of serum neutralizing antibodies and a much higher level of protection in the URT than did recombinant VV xpressing the PIV3 F protein. One possible explanation for the apparent lower immunogenicity of RSV G might be its high carbohydrate content, most of which is likely present in multiple, small, O-linked side chains that might interfere with immune recognition (34, 47).

In tissue culture, monospecific antiserum against the F or HN proteins of simian virus 5 were effective individually in neutralizing the infectivity of released virus, but only anti-F antibodies were effective in preventing viral spread by cell-to-cell fusion (21, 22). As is the case for simian virus 5, rapid and extensive cell-to-cell fusion is a prominent cytopathic effect of PIV3 in tissue culture. These observations suggested that immunity against the HN protein alone might be less protective than immunity against F. However, for PIV3, the observed result (Table 2) was that immunization with either VV recombinant alone was highly protective, and that vaccinia-HN appeared to be more rather than less protective than vaccinia-F.

Attempts at immunoprophylaxis in humans for parainfluenza viruses as a group have met with discouraging results. Field trials with Formalin-inactivated RSV or measles vaccine failed to demonstrate protection against disease (11, 44). Paradoxically, LRT disease of RSV vaccinees and febrile illness and rash in measles virus vaccinees actually was enhanced over that observed in unvaccinated persons during subsequent natural infection (10, 15, 17). Thus, the possibility of disease enhancement after vaccination against parainfluenza viruses is a realistic and serious concern. Recently, RSV disease enhancement in human vaccinees was correlated with an unbalanced immune response, in which vaccinees developed a high titer of circulating antibodies that bound viral protein efficiently (as measured by ELISA) but failed to neutralize viral infectivity effectively (as measured by plaque reduction) (24). In contrast, the serum antibodies induced by the PIV3 vaccinia-F and vaccinia-HN recombinants was balanced with regard to ELISA, HI, and neutralizing titers relative to antibodies induced by viral infection. However, it will be important to resolve the possibility of disease enhancement by direct examination of pulmonary pathology in immunized, challenged animals. This will also resolve the question of whether antibodies against HN in the absence of antibodies against F can induce disease enhancement (21).

PIV3 and RSV disease is most severe in the very young (2 to 8 months of age). Thus, recipients for any PIV3 or RSV vaccine will have significant levels of maternally derived antibody. Because the general population is no longer exposed or generally immune to variola virus or VV (14, 38), maternal antibody should lack anti-VV activity. Thus, VV has a unique advantage as a vector for immunoprophylaxis of the very young. Maternal antibody might also suppress the immune response to the expressed PIV3 or RSV antigens, a potential problem for any parenterally administered vaccine. The use of a replicating agent such as VV recombinants might overcome this effect. The major obstacle to the use of recombinant VV in humans is the occasional occurence of postvaccination complications (1). It remains to be seen whether further developments, such as the reduction in virulence associated with the $TK^-$ phenotype of VV recombinants (2), can reduce these hazards to an acceptable level. Alternately, the use of other recombinant viral vectors, such as adenovirus (7), could be investigated. The VV recombinants described here represent useful reagents for further analysis of host immunity to PIV3 and for testing the general usefulness of live recombinant viruses as vaccines.

## ACKNOWLEDGMENTS

We thank Robert M. Chanock for support and advice, Sekhar Chakrabarti and Bernard Moss for materials and advice in constructing the VV recombinants, Alicia Buckler-White for synthetic oligonucleotides, and Eveline Tierney, Ena Camargo, and Robert L. Horswood for technical assistance.

## LITERATURE CITED

1. **Arita, I., and F. Fenner.** 1985. Complications of smallpox vaccination, p. 49–60. *In* J. Quinnan (ed.), Vaccinia viruses as vectors for vaccine antigens. Elsevier Scientific Publishing Co., Amsterdam.
2. **Buller, R. M. L., G. Smith, K. Cremer, A. L. Notkins, and B. Moss.** 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature (London) 317:813–815.
3. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5:3403–3409.
4. **Chanock, R. M., and K. McIntosh.** 1985. Parainfluenza viruses, p. 1241–1253. *In* B. Fields (ed.), Virology. Raven Press, New York.
5. **Choppin, P. W., and R. W. Compans.** 1975. Parainfluenza viruses, p. 95–178. *In* H. Fraenkel-Conrat and R. R. Wagner (eds.), Comprehensive virology, vol. 4. Plenum Publishing Corp., New York.
6. **Coelingh, K. V. W., C. C. Winter, B. R. Murphy, J. M. Rice, P. C. Kimball, R. A. Olmsted, and P. L. Collins.** 1986. Conserved epitopes on the hemagglutinin-neuraminidase proteins of human and bovine parainfluenza type 3 viruses: nucleotide sequence analysis of variants selected with monoclonal antibodies. J. Virol. 60:90–96.
7. **Davis, A. R., B. Kostek, B. B. Mason, C. L. Hsiao, J. Morin, S. K. Dheer, and P. P. Hung.** 1985. Expression of hepatitis B surface antigen with a recombinant adenovirus. Proc. Natl. Acad. Sci. USA 82:7560–7564.
8. **Elango, N., J. E. Coligan, R. C. Jambou, and S. Venkatesan.**

1986. Human parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein: nucleotide sequence of mRNA and limited amino acid sequence of the purified protein. J. Virol. 57:481–489.
9. Elango, N., G. A. Prince, B. R. Murphy, S. Venkatesan, R. M. Chanock, and B. Moss. 1986. Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recombinant vaccinia virus expressing the RSV G glycoprotein. Proc. Natl. Acad. Sci. USA 83:1906–1910.
10. Fulginiti, V. A., J. J. Eller, A. W. Downie, and C. H. Kempe. 1967. Atypical measles in children previously immunized with inactivated measles virus vaccine: altered reactivity to measles virus. J. Am. Med. Assoc. 202:1075–1080.
11. Fulginiti, V. A., O. F. Sieber, T. J. John, P. Askin, and H. J. Umlauf. 1967. Parainfluenza virus immunization. Pediatr. Res. 1:50–58.
12. Homma, M., and M. Ohuchi. 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural difference of Sendai viruses grown in eggs and in tissue culture cells. J. Virol. 12:1457–1463.
13. Jambou, R. C., N. Elango, and S. Venkatesan. 1985. Proteins associated with human parainfluenza virus type 3. J. Virol. 56: 298–302.
14. Jones, L., S. Ristow, T. Rilma, and B. Moss. 1986. Accidental human vaccination with vaccinia virus expressing nucleoprotein gene. Nature (London) 319:543–544.
15. Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart. 1969. An epidemiological study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. 89:405–421.
16. Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster. Antiviral activity of an antiserum specific for an influenza virus neuraminidase. J. Virol. 2:281–288.
17. Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott. 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. 89:421–434.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
19. Mackett, M., and G. L. Smith. 1986. Vaccinia virus expression vectors. J. Gen. Virol. 62:2067–2082.
20. Markwell, M. A. K., and C. F. Fox. 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. J. Virol. 33: 152–166.
21. Merz, D. C., A. Scheid, and P. W. Choppin. 1980. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J. Exp. Med. 151:275–288.
22. Merz, D. C., A. Scheid, and P. W. Choppin. 1981. Immunological studies of the functions of paramyxovirus glycoproteins. Virology 109:94–105.
23. Murphy, B. R., J. A. Kasel, and R. M. Chanock. 1972. Association of serum anti-nueraminidase antibody with resistance to influenza in man. N. Engl. J. Med. 286:1329–1332.
24. Murphy, B. R., G. A. Prince, E. E. Walsh, H. W. Kim, R. H. Parrott, V. G. Hemming, W. J. Rodriquez, and R. M. Chanock. 1986. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J. Clin. Microbiol. 24:197–202.
25. Nagai, Y., H.-D. Klenk, and R. Rott. 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle Disease virus. Virology 72:494–508.
26. Olmsted, R. A., R. S. Baric, B. A. Sawyer, and R. E. Johnston. 1984. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. Science 225: 424–427.
27. Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins. 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccina virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc. Natl. Acad. Sci. USA 83:7462–7466.
28. Parrott, R. H., H. W. Kim, J. O. Arrobio, J. G. Canchola, C. D. Brandt, J. L. DeMeio, K. E. Jensen, and R. M. Chanock. 1967. Experience with inactivated respiratory syncytial and parainfluenza virus vaccines in infants, p. 35–41. *In* First international conference on vaccines against viral and rickettsial diseases of man. Plenum Publishing Corp., New York.
29. Prince, G. A., R. L. Horswood, E. Camargo, D. Koenig, and R. M. Chanock. 1983. Mechanisms of immunity to respiratory syncytial virus in cotton rats. Infect. Immun. 42:81–87.
30. Prince, G. A., R. L. Horswood, and R. M. Chanock. 1985. Quantitative aspects of passive immunity to respiratory syncitial virus infection in infant cotton rats. J. Virol. 55: 517–520.
31. Prince, G. A., A. B. Jenson, V. G. Hemming, B. R. Murphy, E. E. Walsh, R. L. Horswood, and R. M. Chanock. 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. J. Virol. 57:721–728.
32. Ray, R., V. E. Brown, and R. W. Compans. 1985. Glycoproteins of human parainfluenza virus type 3: characterization and evaluations as a subunit vaccine. J. Infect. Dis. 152:1219–1230.
33. Sanchez, A. and A. K. Banerjee. 1985. Studies on human parainfluenza virus 3: characterization of the structural proteins and *in vitro* synthesized proteins coded by mRNAs isolated from infected cells. Virology 143:45–54.
34. Satake, M., J. Coligan, N. Elango, E. Norrby, and S. Venkatesan. 1985. Respiratory syncytial virus envelope glycoprotein (G) has a novel structure. Nucleic Acids Res. 13: 7795–7812.
35. Scheid, A., L. A. Caliguiri, R. W. Compans, and P. W. Choppin. 1972. Isolation of paramyxovirus glycoproteins: association of both hemagglutination and neuraminidase activities with the larger SV5 glycoprotein. Virology 50:640–652.
36. Scheid, A., and P. W. Choppin. 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology 80:54–66.
37. Schmaljohn, A. L., E. D. Johnson, J. M. Dalrymple, and G. A. Cole. 1982. Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis. Nature (London) 297:70–72.
38. Small, P. A., Jr., G. L. Smith, and B. Moss. 1985. Intranasal vaccination with recombinant vaccinia containing influenza hemagglutinin prevents both influenza virus pneumonia and nasal infection; intradermal vaccination prevents only viral pneumonia, p. 175–178. *In* J. Quinnon, (ed.), Vaccinia viruses as vectors for vaccine antigens. Elsevier Scientific Publishing Co., Amsterdam.
39. Smith, G. W., and L. E. Hightower. 1981. Identification of the P proteins and other disulfide-linked and phosphorylated proteins of Newcastle disease virus. J. Virol. 37:256–267.
40. Spriggs, M. K., and P. L. Collins. 1986. Human parainfluenza virus type 3: messenger RNAs, polypeptide coding assignments, intergenic sequences and genetic map. J. Virol. 59:646–654.
41. Spriggs, M. K., R. A. Olmsted, S. Venkatesan, J. E. Coligan, and P. L. Collins. 1986. Fusion glycoprotein of human parainfluenza virus type 3: nucleotide sequence of the gene, direct identification of the cleavage-activation site and comparison with other paramyxoviruses. Virology 152:241–251.
42. Stanley, J., S. J. Cooper, and D. E. Griffin. 1986. Monoclonal antibody cure and prophylaxis of lethal Sindbis virus encephalitis in mice. J. Virol. 58:107–114.
43. Storey, D. G., K. Dimock, and C. Y. Kang. 1984. Structural characterization of virion proteins and genomic RNA of human parainfluenza virus 3. Virology 52:761–766.
44. Vella, P. P., R. E. Welbel, A. F. Woodhour, C. C. Mascoli, M. B. Leagus, O. L. Ittensohn, J. Stokes, and M. R. Hilleman. 1969. Respiratory virus vaccines: field evaluation of a trivalent parainfluenza virus vaccine among preschool children in families, 1967–1968. Respir. Dis. 99:526–536.
45. Walsh, E. E., J. J. Schlesinger, and M. W. Brandriss. 1984.

Protection from respiratory syncytial virus infection in cotton rats by passive transfer of monoclonal antibodies. Infect. Immun. 43:756–758.
46. Wechsler, S. L., D. M. Lambert, M. S. Galinski, and M. W. Pons. 1985. Intracellular synthesis of human parainfluenza type 3 virus specified polypeptides. J. Virol. 54:661–664.
47. Wertz, G. W., P. L. Collins, Y. Huang, C. Gruber, S. Levine, and L. A. Ball. 1985. Nucleotide sequence of the G protein gene of human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proc. Natl. Acad. Sci. USA 82:4075–4079.
48. Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman. 1985. Rapid and easy sequencing of large linear double stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. 2:89–94.
49. Zoller, M. J., and M. Smith. 1984. Oligonucleotide directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3:479–488.

1: Virology. 1987 Oct;160(2):465-72.

Related Articles, Links

# Expression of biologically active and antigenically authentic parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein by a recombinant baculovirus.

**van Wyke Coelingh KL, Murphy BR, Collins PL, Lebacq-Verheyden AM, Battey JF.**

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892.

The hemagglutinin-neuraminidase (HN) gene of human type 3 parainfluenza virus has been inserted into a baculovirus expression vector under the control of the polyhedrin promoter. HN protein produced in insect cells by the recombinant baculovirus appeared to be glycosylated, was transported to the cell surface, and was biologically active. All of the HN epitopes previously mapped functionally to a region(s) involved in neuraminidase and/or hemagglutination activities were conformationally unaltered on the recombinant protein. The HN produced in this system also induced a protective immune response in immunized cotton rats. From these studies we conclude that the HN expressed in insect cells represents a source of authentic HN glycoprotein suitable for structural analysis and immunization.

PMID: 2821684 [PubMed - indexed for MEDLINE]